

*Mitochondria*. Immo E. Scheffler  
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# **MITOCHONDRIA**

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# MITOCHONDRIA

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**Immo E. Scheffler**

University of California, San Diego



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*To Diana and Omi*

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## PREFACE

When I talked to friends and colleagues about my writing a book on “Mitochondria,” they naturally assumed that I was editing a book about Mitochondria, and the usual follow-up question was in regard to the other contributors. My response, that there were none, caused a pause and a look of incredulity, a reaction that immediately raised doubts in my mind whether I was in over my head. I knew it was going to be a challenge!

There certainly was never any doubt that I would have enough material to include. There are close to 80,000 references with mitochondria as a keyword in the Medline database. That was just one of the challenges: Is it possible to gain command and perspective on such a vast range of studies covering anthropology, biochemistry and biophysics, cell biology, diseases, evolution, forensics, genetics, history, and more. The reader will have to be the judge.

I was “dragged” into the subject more than 25 years ago—several years after I thought I had had my last course in intermediary metabolism. A mutant hunt with Chinese hamster cells yielded auxotrophs for carbon dioxide, which, on further analysis, turned out to be respiration-deficient mutants, the first such mutants identified for mammalian cells in tissue culture. A search of the National Science Foundation files by one of Senator Proxmire’s eager interns in quest of a candidate for the “Golden Fleece of the Month” award uncovered one of my successful grant applications, and I subsequently received a telephone call from the *National Inquirer* asking why I was interested in respiration-deficient Chinese. Several witty answers occurred to me only after I had hung up. Unfortunately, in those days I did not know that just about every neurodegenerative disease, and even aging in general, could potentially be explained with the help of defective mitochondria. The subject has grown on me over the years, and I certainly find it fascinating, even if mitochondrial defects cannot explain all such problems.

One of the most memorable quotes by one of my esteemed undergraduate teachers is: "We learn more and more about less and less until we know everything about nothing." An ever-expanding horizon in the biological sciences makes specialization inescapable and necessary. Accepting the challenge of writing this book was my personal act of rebellion against this trend. Quite obviously, an active participation in research requires the individual researcher to have a focal point. But does that mean that researchers interested in complex V (ATP synthase) no longer talk to researchers investigating complex IV (cytochrome oxidase)? Anecdotal reports from the Gordon Conference on Electron Transport suggest that we are not too far from such a situation.

Mitochondria have been pivotal in the development of some of the most profound ideas in modern Biology. Thermodynamics and life (Bioenergetics) are intricately linked through mitochondria, and the Chemiosmotic Hypothesis is surely one of the great intellectual triumphs of twentieth century science. They occupy a central position in any discussion of metabolism. It is fair to say that the evolution of Cell Biology from Anatomy and Physiology received a major impulse from the visualization of mitochondria by electron microscopy, and from their physical isolation by differential centrifugation. When DNA was discovered in mitochondria, "molecular" biologists took notice and geneticists had to pay attention. The evolution of eukaryotes owes much to the conversion of a prokaryotic symbiont to a subcellular organelle, now known as mitochondria. Today, understanding the evolution of large populations of mitochondrial genomes in somatic cells of a complex organism remains a major challenge: How does it happen and what are the consequences?

A historical perspective and an emphasis on the progression of our understanding of the properties and the role of mitochondria have guided the writing of several chapters. I have tried to give credit where credit is due. Several reviewers pointed out missing references or seminal papers. Nevertheless, no claim is made to have pursued every concept to its roots. Time has obscured many details from the past, and the explosion of knowledge in the past two or three decades has involved many laboratories almost simultaneously. It is relatively easy to identify many giants in the field, but it is not possible for an outsider to identify them in all the areas covered.

This book is intended first of all to remind the reader of the fundamental importance of mitochondria in the understanding of life and the eukaryotic cell, and the aim is to ignite in the serious beginning student an interest in the many fascinating aspects of this organelle. There are also many mature investigators, especially in the medical sciences, but also in other fields, who will have rediscovered mitochondria later in their careers when addressing problems related to diseases such as cardiomyopathy and neurodegenerative disease, developmental processes such as apoptosis, cancer, and senescence, or male sterility in plants. This volume is an attempt to provide such individuals with a single source of information on the major aspects of the biochemistry, cell biology, and molecular genetics of mitochondria. It is hoped that this book will help in establishing a broad perspective on mitochondrial biology and its many facets, and at the same time that it will provide sufficient detail and depth to make it a reference of some lasting value. The reader should be able to find a more than superficial treatment of the various topics with reference to diverse organisms, and use

this treatise and its key references as a convenient springboard into the voluminous past literature, as well as future publications. While more potentially relevant studies are being published as this is being written, they should not make the basic principles and insights obsolete.

There was some thought of limiting the discussion to mitochondria of one organism, for example, mammals. In the future this may be the only way of coping with the wealth of information being accumulated. Clearly, information such as sequence polymorphisms in mitochondrial genomes in vast numbers of representatives of different human ethnic groups will be most efficiently stored and disseminated by the World Wide Web. This treatise is more concerned with the nature of information and data to be collected, using illustrative examples, and it attempts to demonstrate how such data can serve in addressing fundamental biological questions about the role of mitochondria in health and disease, in understanding the evolution of humans and other organisms, and their role as the powerhouse of the fundamental unit of life: the cell.

Model systems with lower eukaryotes and comparative studies have provided invaluable guidance and insights in many aspects of mitochondrial biology. For example, the budding yeast, *Saccharomyces cerevisiae*, has been the organism of choice for numerous pioneering studies. At the same time one should not develop the notion that all problems related to mitochondria or oxidative phosphorylation can be addressed in yeast. It is true that there is a significant amount of conservation of structure and function, especially with regard to electron transport and oxidative phosphorylation. On the other hand, some of the most striking differences between different organisms can be seen in the size of the mitochondrial genomes and in the organization of the genes. Maintenance, repair, recombination, and expression of the different genomes are not nearly as conserved and similar as one might have initially suspected. Undoubtedly there are lessons for the evolutionary biologist, and one may wonder whether important functional characteristics and regulatory mechanisms are also affected. Certainly, the bizarre organization and expression of genetic information in kinetoplasts of trypanosomes, for example, has raised many interesting questions.

This book could serve as a textbook for an advanced graduate course, but it is not written primarily as a "textbook," with a glossary and review questions and exercises. The extensive, but by no means exhaustive, reference list has been assembled to give the interested reader a start in exploring past investigations. In each reference list asterisks indicate recent reviews, books, or book chapters that should be consulted for comprehensive and authoritative treatments of specialized subjects. Of necessity, such sources also had to substitute as references for numerous additional original papers. I apologize for any serious omissions or misattribution of credit.

I owe a debt of gratitude to numerous individuals who were encouraging and helpful in many ways. Several individuals reviewed entire chapters of the book and provided valuable constructive criticisms for many sections. Special thanks and credit should go to Drs. W. Allison, S. Brody, J. Fee, Y. Hatefi, L. Simpson, M. Stoneking, C. Wills, and T. Yagi for their help, encouragement, and many constructive comments. Two anonymous reviewers also provided most valuable and appreciated suggestions for corrections and improvements. Drs. J. Singer, G. Palade, G. Perkins, L. Simpson,



L.B. Chen, and J. Nunnari are owed thanks for contributing original photographs, but I am especially indebted to Dr. D. Fawcett who responded from his retirement in Montana with a generous collection of photographs from his book *The Cell*. The contributors of the original photographs are acknowledged in the Figure legends, and I thank the Publisher, the W.B. Saunders Company, for permission to reproduce these electron micrographs. Dr. Robert Harington, Senior Editor at John Wiley & Sons, originally spotted in me the glimmer of the idea for this book and was most encouraging and helpful in bringing it to fruition. I thank Ms. Luna Han and Kristin Cooke at the Editorial Office for constant attention, invaluable help, and guidance during the final production stages.

I am particularly grateful to R. Chan, of EmbiTec, San Diego, California, whose financial support of the laboratory allowed me to take time out from endless grant applications long enough to get the bulk of this material onto paper. Graduate students and postdoctoral fellows in the laboratory over the years have also contributed in numerous ways: by providing data to think about, by asking challenging questions, and by being an essential and lively part of an environment in which teaching, learning, and research can thrive.

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# 1

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## HISTORY

A review by Cowdry in 1918, quoted by Lehninger [1], contains more than a dozen terms referring to structures we now identify as mitochondria: blepharoblasts, chondriokonts, chondriomites, chondrioplasts, chondriosomes, chondriospheres, fila, fuchsinophilic granules, Korner, Fadenkorper, mitogel, parabasal bodies, plasmasomes, plastochondria, plastosomes, vermicules, sarcosomes, interstitial bodies, bioblasts, and so on. Beginning around 1850, microscopists used the words chondros (Greek), grain (English), and Korn (German) in their descriptions of the morphology of distinct cell structures. Improvements in staining yielded more accurate morphological descriptions, and in some tissues the “grains” were seen as “threads” (*Faden* in German, *mitos* in Greek), hence Fadenkorper or mitochondria (noted by C. Benda around 1898). In 1888, by microdissecting some of these granules out of insect muscle and observing them to swell in water (R.A. Kollicker), microscopists reached the conclusion that the cells had a membrane.

With our current knowledge it is astonishing to learn that in 1890 R. Altman called these granules “bioplasts” in a book on *Elementarorganismen*, in which he proposed that these granules were autonomous, elemental living units, forming bacterial-like colonies in the cytoplasm of the host cell. A wild, lucky guess, or an idea ahead of its time?

In the early part of the twentieth century, cytologists were eager to provide their geneticist colleagues with a concrete entity to which they could assign a role in the transmission of genetic information, and, for a short time, mitochondria were favored. In 1912 and 1913, B.F. Kingsbury and O. Warburg found that these granular, insoluble subcellular structures were associated with respiration, and this function challenged the theory of their role in genetics. But another thirty to forty years of intense and painstaking biochemical analyses were required to lead to the characterization of

mitochondria as the “powerhouse of the cell.” Some of the most illustrious names in the early history of biochemistry can be found among contributors to the evolution of this concept. The discoveries of cytochromes, iron-porphyrin compounds (heme), flavin and pyridine nucleotides, and various dye-reducing dehydrogenases fall into the period between 1920 and the 1930s, and the formulation of the citric acid cycle by Krebs was one of the crowning achievements of the study of metabolism and respiration in muscle preparations.

Although K. Lohmann discovered adenosine triphosphate (ATP) in 1931, it took ten more years to demonstrate its general role beyond muscle, and during this period O. Warburg and O. Meyerhof described what is now referred to as substrate level phosphorylation (ATP synthesis coupled to the enzymatic oxidation of compounds such as glyceraldehyde phosphate), in contrast to oxidative phosphorylation, first shown by H.M. Kalckar to be firmly coupled to respiration (1937–1941).

This was also a time when biochemists proceeded to grind up tissue, filter it through cheesecloth, perhaps even centrifuge the mixture at some indeterminate speed, and then threw away everything but the supernatant. Insoluble particles constituted a nuisance and an insurmountable obstacle in the purification of an enzyme, and students were urged not to “waste clean thoughts on impure enzymes.” The study of mitochondria required the isolation and purification of larger quantities of the organelle, and the first attempts in this direction were doomed by the use of unsuitable buffers and media for cell suspension and breakage.

Cell Biology metamorphosed out of the older science of Cytology when two powerful new methodologies were perfected and applied to the study of biological tissues. Pioneering advances and applications were made in parallel and synergistically at the same institution during the 1940s: at the Rockefeller Institute, A. Claude and his colleagues began to use the centrifuge as a sophisticated analytical tool for the fractionation of subcellular structures (differential centrifugation), a technique which C. DeDuve [2] would later describe as “exploring cells with a centrifuge.” Subcellular particles were fractionated reproducibly and with increasing resolution to achieve pure fractions. At the same time, careful biochemical characterizations were carried out. Important concepts to emerge were the recognition of the polydisperse population of particles (size variation), and the postulate of biochemical homogeneity. Microsomes and mitochondria were represented by overlapping populations of granules of different size, but at the large end were almost pure mitochondria, and at the small end were almost pure microsomes (which were later resolved further into lysosomes and peroxisomes [2]). In 1948 G.H. Hogeboom, W.C. Schneider, and G. Palade discovered that 0.88 M sucrose greatly stabilized mitochondria, which aided their isolation from liver in a morphologically intact form.

The criterion of morphological intactness was practicable only because the groups led by K. Porter and G. Palade used the electron microscope in the exploration of cells. Particles could now be viewed and compared *in situ* and after isolation by differential centrifugation, so that investigators felt confident that the particle was intact, and therefore most likely completely functional.

Nevertheless, isolating and looking at a particle does not immediately give many clues about its biological function, although remarkably prescient guesses and de-

ductions had been made from staining experiments. An approach from a different direction finally led to the full appreciation of the role of mitochondria in respiration. A.L. Lehninger (and independently L.F. Leloir and J.M. Munoz) in the period 1943–1947 had focused on the oxidation of fatty acids in liver homogenates and found the activity to be dependent on an insoluble component that was sensitive to osmotic conditions. The newly established conditions for mitochondrial isolation were applied by E. Kennedy and A.L. Lehninger to prove that fatty acid oxidase activity of the liver was found almost exclusively in mitochondria. The same investigators then extended the biochemical characterization of these organelles by demonstrating that 1) the reactions of the citric acid cycle can be carried out in mitochondria at a rate that can account for most if not all of the activity found in liver cells, and 2) such reactions were accompanied by the synthesis of ATP (oxidative phosphorylation).

Just as a cell was recognized to be much more than a “bag of enzymes,” it soon became clear that many of the enzymes catalyzing the biochemical reactions observed in mitochondria were not simply contained within this organelle in soluble form by the mitochondrial membrane. In fact, the potential complexity of this organelle became apparent from the early electron microscopic observations which revealed the existence of an inner and an outer membrane, with the inner membrane often highly folded (termed *cristae* by G. Palade). Topologically one can therefore distinguish two spaces inside the mitochondrion: the intermembrane space and the matrix. However, a full appreciation of the significance of this compartmentalization was probably not achieved until much later.

The enzymes for fatty acid oxidation and for the citric acid cycle (with the exception of SDH) were found to be soluble in the mitochondrial matrix. The enzymes responsible for oxidation of NADH and electron transport to oxygen were insoluble and localized to the inner membrane (*cristae*). A more detailed description of their characterization is given in Chapters 5 and 6. Here we mention two accomplishments of the 1960s. First, the overall arrangement of the components of the electron transport chain and the flow of electrons from dehydrogenases to flavoproteins to various non-heme iron-sulfur centers and cytochromes and finally to oxygen, first glimpsed by D. Keilin in the 1930s, was established by a combination of spectroscopic studies and the use of specific inhibitors such as rotenone, antimycin, and cyanide in various laboratories. The studies of B. Chance deserve special mention. D.E. Green was another influential investigator in the 1950s and 1960s. While his ideas about an “elementary particle” within mitochondria have not stood up to the test of time, his Institute for Enzyme Research was the training ground for a number of prominent researchers in the subsequent decades. A very informative, entertaining, and highly personalized account has been written by one of the pioneers in the field, E. Racker [3]. Second, the efforts of Y. Hatefi and colleagues [4,5] culminated in the fractionation and characterization of five multisubunit complexes from the inner membrane: four are involved in the respiratory chain, and the fifth was identified as the site of the phosphorylation of ADP to ATP. Among the current high points in the biochemical and structural analysis of these complexes is undoubtedly the recent achievement of high-resolution structures for complex III [6], complex IV (cytochrome oxidase) [7] and the  $F_1$  component of complex V (ATP synthase) [8–10] by x-ray diffraction and other biophys-

cal means. A Nobel Prize in Chemistry has just been awarded for the elucidation of the structure and mechanism of the  $F_1$ -ATP synthase to J. Walker of Cambridge University and P. Boyer at the University of California at Los Angeles.

The distinction between substrate level phosphorylation and oxidative phosphorylation is the subject of examination questions for thousands of undergraduate students every year. The former is straightforward to explain in terms of enzyme kinetics and the coupling of exergonic and endergonic enzyme-catalyzed reactions. The challenge to explain oxidative phosphorylation has preoccupied some of the best biochemists for a good part of their career. An ingenious solution, offered by P. Mitchell [11], was slow to be accepted, but it eventually revolutionized our thinking about bioenergetics, membranes, membrane potentials, active transport, ion pumps, and "vectorial metabolism." A detailed understanding of the structure of membranes, and the relationship between lipids and integral membrane proteins, was a prerequisite [12]. The chemiosmotic hypothesis has found universal acceptance not only in explaining oxidative phosphorylation in mitochondria, but also aspects of photosynthesis in chloroplasts, and light-driven phosphorylations in bacteria.

While failing to live up to early expectations of mitochondria as the carriers of all hereditary information, a most important discovery was made in 1963 when one of the first definite identifications of DNA in mitochondria was made [13]. This discovery had in many ways been anticipated by the discovery of nonmendelian, cytoplasmic inheritance in yeast by the Ephrussi laboratory [14]. Ramifications of this discovery are wide. It renewed and strengthened interest in the evolutionary origin of mitochondria. The problem of understanding how two genomes, nuclear and mitochondrial genes, interact in the biogenesis of this organelle is still an acute one. In vertebrates the mitochondrial genome is relatively small, deduced from isolated circular mtDNA molecules visualized by electron microscopy. The complete sequencing of a mammalian mtDNA in Cambridge was one of the earliest triumphs of the DNA sequencing technology invented by F. Sanger and his colleagues in the 1970s [15]. Almost simultaneously, G. Attardi [16] and his coworkers at the California Institute of Technology determined the nature of the transcripts derived from this genome and set the stage for the identification of all the genes encoded by mammalian mtDNA. Changes in mitochondrial DNA sequences are believed to represent a molecular clock on a time scale that appears particularly relevant for human evolution, and provocative ideas and speculations have centered around deductions from such sequence comparisons, with implications for primate and human evolution ("the Mitochondrial Eve"), and the spread of human populations by global migrations. Controversies still center around the question whether this clock is more accurate than a sundial (e.g., N. Howell). Currently, forensic investigations utilize information about mitochondrial DNA sequences from victims and suspects. Finally, where there is DNA, there must be mutations. An explosion of publications in the last decade, triggered by the pioneering work of Wallace and his colleagues [17] and Holt and colleagues [18], has described mutations in mitochondrial DNA that are directly responsible for human genetic diseases (myopathies and neuropathies), while speculations go even further in relating accumulating defects in mitochondrial DNA to a variety of ailments accompanying aging and senescence.

Thus, mitochondria occupy a central position in our understanding of the cell, the “basic unit of life,” and the study of mitochondria has, from the very beginning, revealed not only details, but also fundamental insights covering the entire spectrum from biophysics to cell biology and genetics. Their demonstrated role in disease, and their implied role in neurodegenerative developments in advanced age makes them particularly fashionable now, but a history of biochemistry is unthinkable without reference to mitochondria and the monumental discoveries made in the course of their study. Similarly, the characterization of mitochondrial morphology by electron microscopy in intact cells and tissues and after cell fractionation (e.g., by G. Palade and F.S. Sjostrand and coworkers) coincided with and even triggered the emergence of Cell Biology as a distinct science. The discovery of these and other organelles emphasized the compartmentalization of the cytosol, and hence forced investigators to focus on exploring the various mechanisms of protein targeting to distinct organelles. Today, one of several frontiers is the integration of mitochondria into the cell and their distribution in the cytosol by means of their interaction with the cytoskeleton, especially in various highly differentiated cells. Many questions remain, and an understanding of the achievements described in the following chapters will help to define future directions.

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# 2

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## EVOLUTIONARY ORIGIN OF MITOCHONDRIA

We can't rewind the tape of life and replay it to see what happens next time, alas, so the only way to answer questions about such huge and experimentally inaccessible patterns is to leap boldly into the void with the risky tactic of deliberate oversimplification.

—from *Darwin's Dangerous Idea: Evolution and the Meanings of Life* by Daniel C. Dennett

Soon after mitochondria were first glimpsed in cells of higher organisms about 120 years ago, the idea that they were somehow related to bacteria was expressed in a more or less explicit form by R. Altmann (1890), K.C. Mereschkovsky (1910), P. Portier (1918), J.E. Wallin (1927), and J. Lederberg (1952) (see Reference 1 for a complete bibliography). The idea continued to float about without much further experimental stimulus for several decades, until the discovery of DNA in this organelle gave it a major boost. Then, thoughts about the origin of mitochondria and chloroplasts culminated in an extremist statement of the Serial Endosymbiotic Theory, that "The eukaryotic 'cell' is a multiple of the prokaryotic 'cell'" (F.J.R. Taylor, 1974), and L. Margulis became a particularly eloquent and forceful popularizer of ideas on symbiosis in eukaryotic cellular evolution. In 1925 such ideas had been dismissed as "too fantastic for present mention in polite biological society" (E.B. Wilson, 1925). An anonymous review of a book on the subject by P. Portier appeared in *Nature* and contained the following: "... Prof. Portier is good-humored enough to quote the paradox that a theory is not of value unless it can be demonstrated to be false. We have no hesitation in prophesying that his theory will attain that value. . . ." Today, while there may still be snickering in "polite biological society" about the proposed derivation of eukaryotic cilia and flagella from symbiotic spirochetes, the prokaryotic ancestry of mitochondria is discussed in most textbooks, including those written by the "estab-



lishment.” Formally it is necessary to consider at least two alternative hypotheses concerning the evolutionary origin of mitochondria. The autogenous origin hypothesis (direct filiation) proposes that mitochondria arose from within a single cell by a process of intracellular compartmentalization and functional specialization. When DNA was discovered in these organelles, the assumption had to be added that genetic information from a single nuclear genome became distributed between these two compartments. The second hypothesis suggests that mitochondria originated from a symbiotic relationship between a protoeukaryotic cell and a primitive prokaryote capable of oxidative phosphorylation. Only the second hypothesis is seriously considered today.

In 1982, Gray and Doolittle’s critical review of the arguments for and against the endosymbiont hypothesis [2] made an attempt to define the kind of data on which proof for the endosymbiont hypothesis must rest, and it concluded that “the case for an endosymbiont origin of mitochondria was more problematic and considerably less certain” than the case for the origin of chloroplasts (plastids) from photosynthetic eubacteria (cyanobacteria). A huge volume of additional data began to accumulate during the 1980s, which was periodically reviewed by Gray and others, culminating in an exhaustive and authoritative review and evaluation by Gray in 1992 [3]. Gray concluded that reevaluating alternate hypotheses was pointless in view of the massive and compelling evidence in favor of the endosymbiont hypothesis.

The greatest boost to the hypothesis of the endosymbiotic origin of mitochondria came from the discovery of DNA in mitochondria [4], and from the generalization that all mitochondria in all eukaryotes contained DNA (exceptions to this proposition are discussed later in this chapter). Another statement, that all eukaryotes contain mitochondria, is also not completely tenable, since a group of eukaryotes placed in the kingdom of Archezoa [5] lack mitochondria and may represent surviving relatives of the earliest eukaryotes. Before the genetic information in mtDNA was completely analyzed, it was shown that mitochondrial DNA could be mutated, and that such “cytoplasmic” mutations had serious consequences—not surprisingly, affecting the capacity of the cells to carry out oxidative phosphorylation and respiration [6]. Thus, the functional importance of this genome was proved. These early genetic investigations were primarily performed with the yeast *Saccharomyces cerevisiae*, and many others have followed. Mitochondrial DNA encoded very specific and unique genes, although it was obvious almost from the start that the mitochondrial genome was far too small to encode information sufficient for a free-living organism. The loss of genetic information from primordial protomitochondria was compensated by functions encoded by the nucleus, but at this point one may have to distinguish between two possibilities. An obvious one is that protomitochondrial genes were transferred to the nucleus, and there is persuasive evidence for such events to have occurred. It should also be obvious, however, that symbiosis implied that the bacteria destined to become mitochondria had to invade a host cell, a protoeukaryote, which, before this evolutionary event, was equally capable of an independent existence. The distinction between the two cells in the simplest view is that the protoeukaryote already had its DNA compartmentalized in a nucleus with a nuclear membrane, while the protomitochondrion resembled a bacterium without a nucleus. One can speculate that the

free-living protoeukaryote had the capacity for anaerobic glycolysis and fermentation, while the prokaryote contributed the electron transport system. Both must have been capable of DNA replication, transcription, protein synthesis, and the biosynthesis of various lipids to form a membrane. Krebs cycle enzymes may also have been present in both for the purpose of interconverting short carbon compounds and amino acids. Therefore, the initial association between these two cells must have led to a considerable amount of redundancy of genetic information. The redundant genes in the mitochondrion could simply be lost, others were transferred to the nucleus, and only a very small number had to remain in the mitochondrion.

Gray [3] affirmed that it is absolutely necessary to start with the presumed common ancestor of both eukaryotic and prokaryotic cells, and then to consider distinct traits (genes) that evolved specifically in one of the groups. Only these unique and novel genes contribute to the symbiotic relationship and distinguish the symbiont from the host.

It is not my intent here to speculate about the origin of life and the minimal number of genes required for an independent existence, regardless of the ultimate energy source. Arguments similar to the above apply to the hypothesized origin of chloroplasts (plastids) from photosynthetic bacteria. Light as an energy source has been available since the beginning of the earth, and there is a consensus that the evolution of photosynthesis preceded respiration. Respiration requires oxygen, and oxygen did not become abundant until massive quantities were liberated by photosynthesis. If photosynthesis was performed exclusively by prokaryotes during this early period of life on earth, it is possible to speculate that mitochondria were acquired by a eukaryote before the acquisition of photosynthetic prokaryotes, which subsequently evolved into chloroplasts in plants. Thus, the mitochondria of plants, animals, and fungi may all have a common ancestor and hence similar properties.

The arrival of oxygen on earth was not an unmixed blessing. Apart from the fact that uncontrolled respiration is combustion and “fire” with all of its destructive aspects, even a highly controlled reaction such as respiration can generate “reactive oxygen species” such as the hydroxyl radical and hydrogen peroxide, whose potential for destruction of biological macromolecules may provide a basis of an explanation for an age-related deterioration of the mitochondrial genomes and perhaps even for senescence and death [7]. According to F. Jacob “the most important inventions of evolution are sex and death.” Did death from free-radical bombardments precede programmed cell death (now called *apoptosis*)? Does the mechanism of apoptosis require mitochondrial functions? (See Chapter 7.)

It is more fruitful to pursue the molecular fossil record for a protomitochondrion. All mitochondrial genomes encode two ribosomal RNAs and most encode 20 or more tRNAs (a notable exception may be protozoans such as trypanosomes). Mammalian mtDNA also encodes 13 peptides for complexes of the OXPHOS system, and additional mitochondrial genes are found in other organisms (see Chapter 4 for a detailed discussion). From the point of view of evolution it is particularly illuminating to consider the four genes for complex II: they are all nuclear in mammals, but diverse organisms have been found recently in which the mtDNA still carries one or more of the same genes. The iron protein, Ip, and the flavoprotein, Fp, of complex II constitute the

enzyme succinate dehydrogenase (SDH) of the citric acid cycle. SDH is attached to the inner mitochondrial membrane by means of two integral membrane proteins, or anchor proteins,  $C_{II-3}$  and  $C_{II-4}$ . Ip and Fp are remarkably well conserved in evolution at the protein sequence level and even at the nucleotide sequence level from prokaryotes to eukaryotes. This has made it possible to identify and clone the corresponding genes from many organisms, starting with bacteria. As a first approximation and from a biochemist's simplest perspective, the complex in *Escherichia coli* looks very much like the complex in mammalian mitochondria. In bacteria such as *E. coli*, the four genes form an operon and are coordinately expressed. In mammals, the four genes (SDHA, SDHB, SDHC, and SDHD) are dispersed over various chromosomes. The SDHB (Ip) and SDHC ( $C_{II-3}$ ) genes are found on the mitochondrial genome in the red algae *Chondrus crispus* [8,9], and three of the four genes (SDHB, SDHC, and SDHD) are found on the mitochondrial genome of another red alga, *Porphyra purpurea*, and on a phylogenetically distant heterotrophic zooflagellate, *Reclinomonas americana* [10]. Apparently, the transfer of these genes from the mtDNA to the nuclear genome has not (yet) occurred, and these organisms represent examples of "intermediates" in the evolution of a maximally reduced mitochondrial genome. In full agreement with the same train of thought, *Chondrus crispus* mtDNA still retains four genes for ribosomal proteins, while certain plant mtDNAs encode even more ribosomal proteins. In vertebrates all mitochondrial ribosomal protein genes have been translocated to the nucleus. Studies such as these have provided supporting arguments in favor of a monophyletic origin of mitochondria [10]. The diversity of genes still found in mitochondrial genomes can be explained by a random gene loss over time from a common ancestral genome.

When the complete sequence of the mitochondrial genome of the freshwater protozoon *Reclinomonas americana* was recently completed, some rather surprising findings strengthened the hypothesized link between mitochondria and eubacteria-like endosymbionts ("the mitochondrion that time forgot") [11,12]. With 69,034 basepairs the genome is not exceptional in size, but the existence of at least 97 genes on this genome was unexpected, since many of the other mitochondrial genomes now known have far fewer genes, even when their size was an order of magnitude greater (see Chapter 6). The *Reclinomonas* mt genome encoded 23 components of the electron transport chain and oxidative phosphorylation apparatus (compared to 13 in mammals), 18 mitoribosomal proteins (none in mammals, some in plants), 27 distinct tRNAs, 3 ribosomal RNAs, including a 5S RNA not found in mammals, an RNA component of ribonuclease P (used in RNA processing (see Chapter 4), and 4 genes of a multisubunit RNA polymerase resembling a eubacterial-type polymerase. The latter finding is particularly intriguing and unique so far. A preliminary interpretation is that a eubacterial multisubunit RNA polymerase may have been lost during evolution from all other mitochondria (analyzed so far), and replaced by a single-subunit enzyme, encoded by the nuclear genome, with similarities to the T3/T7 enzyme (see Chapter 4).

Ribosomal RNA sequence comparisons have served as a predominant source of data in the construction of phylogenetic trees and evolutionary relationships, and in 1987 C.R. Woese referred to rRNAs as the "ultimate molecular chronometer." These

ancient, ubiquitous and functionally identical molecules and their genes have helped to reevaluate taxonomic subdivisions between existing life forms on earth. It is not the intent here to enter the debate about how many kingdoms there should be (the eukaryotes—Animalia, Plantae, Fungi, Protista; and the prokaryotes—Eubacteria and Archaeobacteria), but to emphasize a major distinction between archaeobacteria, eubacteria, and eukaryotes. Various analyses have shown a closer relationship of archaeobacteria to eukaryotes, and many similarities in genome organization and gene expression between plastid (chloroplast) and eubacterial genomes, in support of the endosymbiotic origin of plastids. A case for a eubacterial origin of mitochondria is more difficult to make on the basis of the “molecular biology as practiced in mitochondria” [3], but rRNA sequence analyses strongly favor the same conclusion. Various aspects, such as information content and organization of the genome, transcription, and translation, are covered in separate chapters when evolutionary comparisons will be instructive, but not central to the discussion.

Mitochondria were found to have their own machinery for protein synthesis. A full treatment of this subject is presented in Chapter 4. However, a frequently quoted example in favor of the endosymbiont hypothesis can be mentioned here. Cytoplasmic protein synthesis employs larger ribosomal subunits with more protein subunits compared to mitochondrial ribosomal subunits, which in turn resemble bacterial ribosomes [13,14]. Cycloheximide is a specific inhibitor of cytoplasmic protein synthesis, whereas chloramphenicol inhibits the bacterial and mitochondrial translation machinery [15]. The distinction based on the differential sensitivity to these two inhibitors has been the basis for many elegant experiments elucidating the specific mitochondrial translation products. On the other hand, a closer look at these similarities in ribosomal properties in bacteria and mitochondria made it apparent that they were rather superficial, hiding the uniqueness and variability of mitochondrial ribosomes (for a detailed discussion see Gray and Doolittle [2]).

Phylogenetic trees based on rRNA comparisons (for the small as well as for the large subunit) not only have proved to be convincing in relating plant mitochondria to eubacteria, but also have helped to identify the living eubacteria that are the closest relative of mitochondria: members of the alpha subdivision of purple bacteria had been suspected initially on biochemical grounds, and rRNA sequence comparisons strengthen this conclusion considerably. By contrast, similar comparisons with animal, ciliate, and fungal mitochondrial rRNA sequences are less convincing because of significant primary sequence divergences. Nevertheless, the eubacterial ancestry of mitochondria is not seriously challenged by such data. Left open is the question of the monophyletic origin of mitochondria, that is, whether the symbiosis between protoeukaryotes and a eubacterial species was a singular event in evolution, or occurred more than once. In particular, did plant mitochondria arise independently of animal mitochondria, for example? The absence of a common root for current rRNA trees had been interpreted by some researchers in support of a biphyletic or multiphyletic origin (see Reference 3 for a summary of the evidence and interpretation), but the same authors also raised the possibility of artifactual trees generated by the choice of algorithms, the selection of sequences, and other methodological subtleties. The most recent studies on complex II genes in the red algae *Porphyra purpurea* and the zoofla-

gellate *Reclinomonas americana* have favored a monophyletic lineage for mitochondria [10].

Has the limit been reached in the reduction of the mitochondrial genome [16]? It is generally agreed that the remaining peptides encoded by mtDNA are extremely hydrophobic peptides, which would not be readily imported by the mitochondrial translocation machinery. On the other hand, two very hydrophobic anchor peptides of complex II are encoded by nuclear genes in most organisms examined so far. Theoretical arguments have been advanced predicting that the mitochondrial genome ultimately need retain only two genes encoding peptides in addition to the ribosomal RNAs and tRNAs [17–19]. The two genes singled out are the apocytochrome b and the COX1 genes encoding peptides with more than 3 or 4 hydrophobic transmembrane segments. Claros and colleagues [18] define a property referred to as mesohydrophobicity (average hydrophobicity over an extended stretch of a polypeptide chain) to make quantitative predictions. The hypothesis was also tested by constructing a series of chimeric genes expressed in the nucleus yielding cytoplasmically synthesized peptides. These peptides had a mitochondrial targeting sequence, a reporter enzyme, and one or more of the eight transmembrane segments of apocytochrome b. A complete interpretation of these results, however, requires also a more detailed understanding of how such integral membrane proteins with multiple transmembrane segments are localized in the inner mitochondrial membrane, an understanding that is still expanding today (see Chapter 4).

Some recent discoveries provide convincing evidence that the complete loss of the mitochondrial genome has in fact occurred in evolution, raising several new questions [16]. Would such mitochondria still be able to support respiration? What would be their function if oxidative phosphorylation was no longer observed? And how would such an organelle still be recognized as a mitochondrion? An emerging consensus is that several phylogenetically distinct lineages of eukaryotes may have undergone a reductive evolution in the course of adapting to anaerobic or micro-aerobic environments. The best studied example is the parasitic flagellate protist *Trichomonas vaginalis*, an air-tolerating anaerobe that has an energy-producing organelle referred to as a hydrogenosome. Hydrogenosomes have been identified in a wide variety of organisms (rumen-dwelling ciliates, fungi, free-living ciliates), and by several criteria such hydrogenosomes are believed to be highly modified mitochondria. They lack DNA, are devoid of cytochromes and most citric acid cycle enzymes, and produce hydrogen by the use of enzymes usually found in anaerobes (pyruvate:ferredoxin oxidoreductase, hydrogenase). They make ATP by substrate level phosphorylations involving succinyl CoA synthetase, a Krebs cycle enzyme. However, they have a double membrane, they import proteins posttranslationally, and they divide by fission. The most persuasive evidence comes from the characterization of *Trichomonas* nuclear genes of the heat-shock proteins Hsp10, Hsp60, and Hsp70 [20]. These proteins are found in the hydrogenosome, and by phylogenetic analysis these Hsps have been found to be among the “most reliable tracers of the eubacterial ancestry of both the mitochondrion and the chloroplast” [16]. Thus, the evidence supports a common origin for hydrogenosomes and mitochondria, rather than the hypothesis of an independent endosymbiotic event involving an anaerobic eubacterium yielding a hydrogenosome.

In conclusion, the argument over the origin of mitochondria appears to have been settled with overwhelming evidence favoring the endosymbiont hypothesis and a monophyletic origin of mitochondria in all eukaryotes. It is likely that as more and more exotic species in remote niches of the living world are explored, additional “mitochondria that time forgot” will be discovered with unusual complements of genes. Such evidence may strengthen arguments over which genes were originally present in the protomitochondria. However, at this time the more challenging questions to ask are questions about the evolution of more individual differences between organisms: the size of the mitochondrial genome, the organization of the remaining genes, and some rather unusual mechanisms in gene expression. Plants have mitochondrial genomes that are one to two orders of magnitude larger than metazoan mitochondrial genomes, without a proportionate increase in the number of genes. What is this “junk DNA” doing and where did it come from? Mitochondrial plasmids and transposonlike parasitic DNA have invaded even mitochondrial genomes. Understanding the evolution of the mind-boggling RNA editing process in trypanosomes (see Chapter 4) is a major challenge by itself. And finally, mitochondrial DNA sequences continue to be altered by mutations, providing an ever richer data base for evolutionary studies—not about the evolution of mitochondria per se, but about the much more recent evolution of closely related species such as the primates, including humans (see Chapter 8).

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# 3

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## STRUCTURE AND MORPHOLOGY: INTEGRATION INTO THE CELL

### 3.1 STRUCTURE AND MORPHOLOGY

When the nineteenth-century morphologists, with their light microscopes, discovered grains (chondria) and filaments (mito), they could not be sure that they were all looking at the same functionally distinct structure in different cells, and undoubtedly they were not. Nevertheless, variability in number and shape must have been apparent. A more systematic approach became possible when selective staining methods were used: in 1890 R. Altman used fuchsin; in 1898 C. Benda used crystal violet and L. Michaelis used Janus green. The capacity of mitochondria to reduce Janus green, an indicator of oxidation-reduction reactions, should have been a major clue, which was evidently missed at the time or, rather, not interpreted fully.

Major progress in the characterization and understanding of the morphology and ultrastructure of mitochondria was not possible until the techniques for electron microscopy and specimen preparation were perfected, but the use of the light microscope in the study of mitochondria was revived in the past few decades by two technical innovations. First, mitochondria could be viewed with the fluorescence microscope in permeabilized cells after staining with specific antibodies and secondary antibodies conjugated to fluorescent dyes. Another very important discovery was the observation that certain rhodamine derivatives and other lipophilic “vital dyes” would stain mitochondria because the dye became concentrated in these organelles by an uptake mechanism driven by the membrane potential [1]. The biophysical basis of this phenomenon is addressed in Chapter 5. Rhodamine staining can be performed with live cells, and, in combination with time-lapse photography and, more recently, image intensification and videotaping, it has opened up a window on the dynamic behavior of mitochondria, including their distribution in the cytosol and their redistribution



after certain perturbations of the cytoskeleton, as well as changes in size and shape. While the resolution of the light-fluorescence microscope is limited, it does offer a view of the entire cell and the global distribution of mitochondria within it (see Section 3.2 for further discussion).

Mitochondria have no fixed size, but typically in hepatocytes and fibroblasts they have a sausagelike shape with dimensions of 3–4  $\mu\text{m}$  (length) and approximately 1  $\mu\text{m}$  (diameter); as a first approximation they do in fact resemble small bacteria in size and shape. The number of mitochondria per cell is a parameter that appears to vary significantly from cell type to cell type, and this will become an important consideration when mitochondrial biogenesis is considered in the context of cell differentiation and specialization (see also Section 3.2). Estimates from serial sections of cells yield values in the range of a few hundred to a few thousand per cell, for example, 800 mitochondria per hepatocyte. Specialized vertebrate cells show significant variations. The human oocyte is estimated to contain 100,000 mitochondria, while spermatozoa have relatively few (but these may be exceptionally large). A somewhat simplified view of mitochondria in some sperm cells is that of a small number of highly elongated mitochondria wrapped around the axoneme of the sperm tail. In lower eukaryotes the number can also be substantial, for example, 500,000 mitochondria have been found in the giant amoebae *Chaos chaos*. The actual number is most likely not the most relevant parameter to consider. Of far more significance may be the mitochondrial volume as a fraction of the cellular volume, or even the total surface area of the mitochondrial cristae per cell (see below). Clearly, details in the morphology depend on the organism. Another number of interest from a geneticist's point of view is the number of mitochondrial genomes per cell. This subject is discussed in detail in Chapter 7, but it can be mentioned here that there are multiple mtDNAs per mitochondrion, and therefore thousands of mtDNAs per cell.

Morphological studies of trypanosomes drew attention to a structure at the base of the flagellum referred to as *kinetoplast*. Seventy years ago the kinetoplast was found to be stainable with Janus green (oxidation–reduction) and shortly thereafter, to give a positive Feulgen reaction (nucleic acid). Later, electron-microscopic examination revealed cristae similar to those found in mitochondria, and the kinetoplast was recognized to be a highly specialized mitochondrion. In 1960, M. Steinert was first to report the finding of DNA in this organelle, but the kinetoplast was considered to be so specialized and exceptional that the finding was not immediately generalized to all mitochondria.

A new chapter in the study of the morphology of mitochondria began with the perfection of the electron microscope as a tool for biological studies. The design of the microscope was a physics and engineering problem. The preparation of a sufficiently thin specimen, a slice of a single cell many times thinner than the diameter of the cell, was a challenge that required a more empirical approach. In sum, the tissue had to be fixed, dehydrated, embedded “in plastic,” stained, and sectioned to give a specimen that could hold together in a high vacuum, provide contrast in the final image, based on differential diffraction of the electron beam (uptake of electron-dense stains such as uranyl acetate and osmium tetroxide by different constituents of the cell), and still reflect biological reality, that is, meaningful images of subcellular structures. The number of skeptics was rapidly diminished by the spectacular images produced by the pioneers at the Rockefeller Institute. A new view of the morphology of individual mi-

tochondria emerged from the studies of G. Palade and his colleagues, and of F.S. Sjostrand, who not only confirmed that mitochondria are membrane-bound organelles, but recognized that there were two membranes: an outer membrane and an inner membrane that was convoluted and folded into *cristae mitochondriales* (G. Palade). It should also be reemphasized that the development of buffers for cell fractionation studies yielding highly purified, intact mitochondria was an important contribution. Such isolated organelles could be viewed by electron microscopy and shown to have a similar morphology to those viewed in situ (Figures 3.1 and 3.2).



**Figure 3.1** Mitochondrion from centroacinar cell of pancreas ( $\times 190,000$ ). (Courtesy of Dr. G. Palade.)

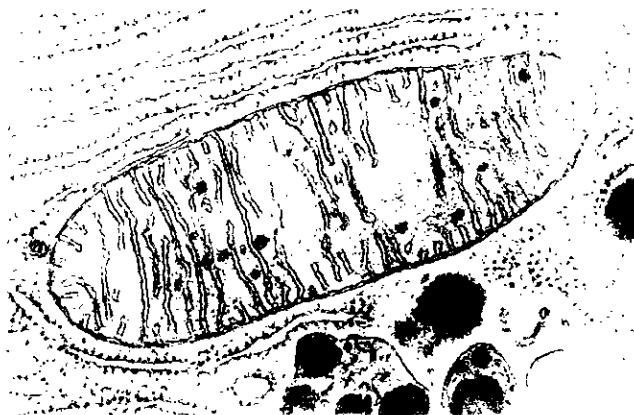


**Figure 3.2** Schematic view of mitochondrion showing the outer and inner membranes and the folded cristae. (Drawing provided by Dr. D. Fawcett.)

A plausible explanation at the time was that this topology vastly increased the surface area of the inner membrane. The significance of the compartmentalization into an intermembrane space and a matrix could not be fully appreciated until later, but in this chapter attention will be largely confined to a discussion of observable morphology of individual mitochondria. It is evident also that the size and shape of mitochondria seen by electron microscopy depends on the plane of sectioning relative to their long axis.

As more examples of mitochondria in different tissues were examined, distinguishing features soon became apparent. The matrix was not homogeneous and exhibited a "fine granularity," and frequently there were distinct crystalline inclusions or small particles of high electron density. Their number could in some instances be shown to depend on the metabolic state of the tissue observed. The high electron density may be the result of a sequestration and storage of calcium. Other studies have suggested that the electron density is the consequence of the retention of osmium tetroxide by a phospholipoprotein that may bind calcium *in vitro*, but may not have such a role *in vivo* (Figure 3.3).

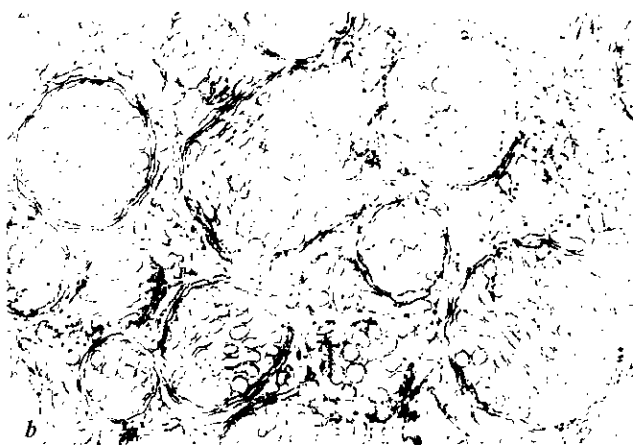
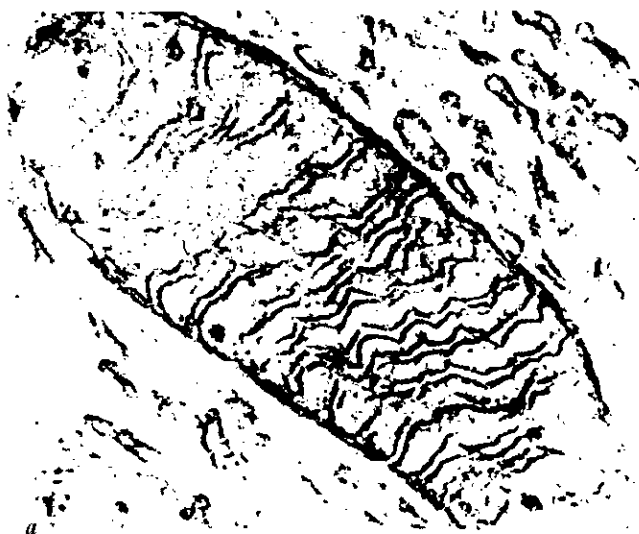
Most notable was the variable appearance of the cristae. They were often lamellar in appearance, but also tubelike in other cells, and a complete description required many images and, ideally, serial sections. Thin sections in the electron microscope still require an interpretation in three dimensions, and frequently the continuity of a membrane is not seen in a given section. Thus, tubes become "free" circular (vesicular) membranes in cross section, and sheetlike foldings of the inner membrane in some sections may appear as flattened sacs which appear to float in the matrix (Figure 3.4). In the current view the inner membrane is continuous, and there appears to be no functional reason to have membrane vesicles within the matrix analogous to the grana of chloroplasts.

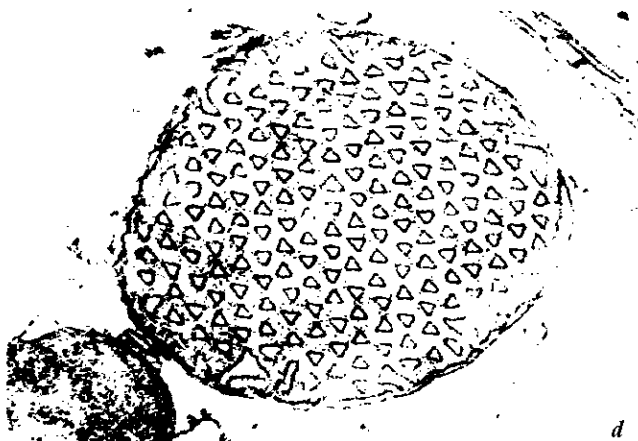
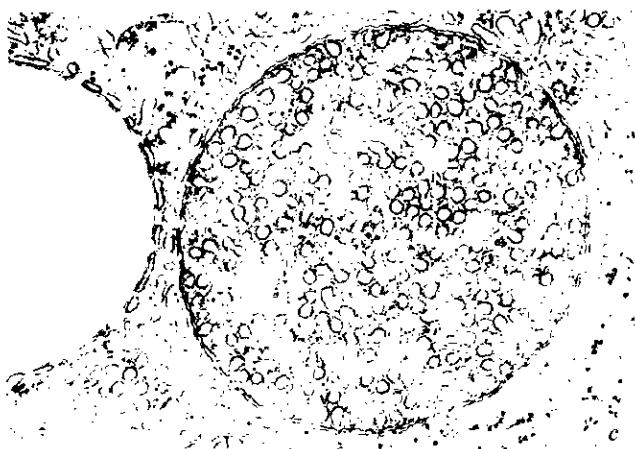


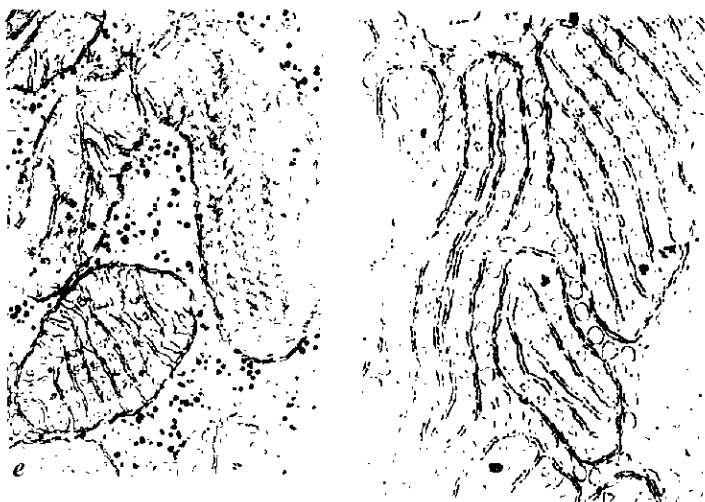
**Figure 3.3** Mitochondrion showing electron-dense inclusions. (From Reference 2, with permission.)

The interpretation of cristae topology from thin-section electron microscopy by G. Palade and E.S. Sjostrand postulated that the cristae are sheetlike invaginations of the inner membrane into the matrix of the mitochondrion (Figure 3.2). A maximum number of cristae can be accommodated if the sheetlike membranes are arranged in parallel, but it seems that the models fudge the issue of what happens to the image if a different plane of sectioning was presented. The T-shaped junction cannot be continuous around the entire circumference of the mitochondrion. Recent advances in electron tomography have made it possible to view complex three-dimensional objects like mitochondria in thick sections and reconstruct a high-resolution three-dimensional image. Data are collected by recording two-dimensional images over a wide range of tilt angles, and three-dimensional reconstructions are achieved by computer-assisted imaging. A three-dimensional resolution in the range of 5–10 nm can be achieved, and hence images of the outer and inner membrane of mitochondria can be viewed at a hitherto unobtainable resolution [3,4]. Only a limited number of tissues have been examined by this powerful methodology (Figure 3.5).

Of particular interest and significance is the conclusion by Perkins and coworkers [3] that cristae consist of a lamellar portion similar to that envisioned before, but the continuity with the inner boundary membrane (which lies close to the outer membrane) is achieved by a relatively small number of narrow tubelike connections. In other words, though continuous, the inner membrane juxtaposed to the outer membrane and the inner membrane of the lamellar structures are connected by a narrow "bottleneck," called a cristae junction. Topologically the intermembrane space is still continuous with the "inside" of the lamellae, but Perkins and his colleagues raise the

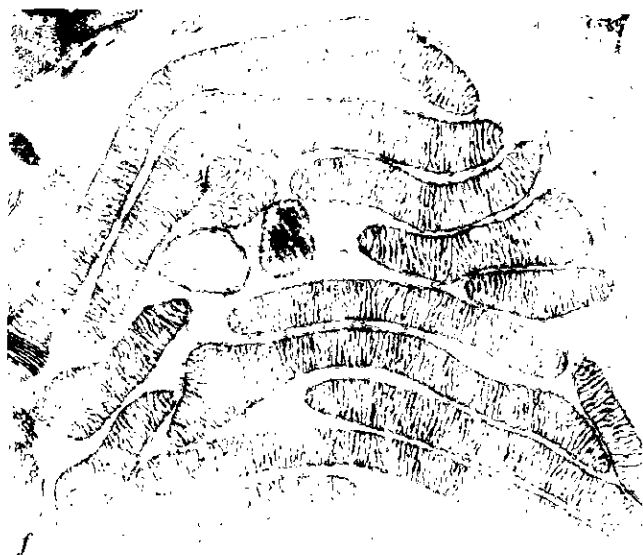


*d*



question whether the bottleneck can constitute a barrier and hence serve to separate two compartments: intracristal and intermembrane. It may also serve to segregate patches of inner membrane with differing protein compositions. Clearly, more studies are necessary to explore the functional implications of these morphological observations.

The number and morphology of cristae is likely to reflect the response of the mitochondria to the energy demands of the cell. Highly folded, lamellar cristae with a large surface area are typically found in muscle and neurons, where the respiratory rate is the greatest. These observations clearly raise questions about the control of cristae formation and the expression of both nuclear and mitochondrial genes encoding mitochondrial proteins. A telling example, although perhaps an extreme one, comes from the study of mitochondrial morphology in the yeast *Saccharomyces cerevisiae*. When grown in the presence of a nonfermentable substrate, such as glycerol or ethanol, the cells are dependent on oxidative phosphorylation, and hence they have mitochondria easily recognizable even by the nonexpert. When grown in the presence of glucose, the biogenesis of the inner membrane is severely repressed, and the morphology, but not the number of the mitochondria, changes dramatically. The details of the mechanism of “glucose repression” of respiration are presented in Chapter 4.2. A similar change in the abundance and morphology of an intracellular organelle can be observed for peroxisomes, for example in the yeast *Picchia pastoris*, where growth on methanol or fatty acids induces the appearance of large peroxisomes. Free-living microorganisms are exquisitely attuned to their environment and capable of adjust-



**Figure 3.4** A: Mitochondria with angular cristae from ventricular cardiac muscle. B: Mitochondria with tubular cristae in amoeba. C: Mitochondria from adrenal cortex. D: Mitochondrion from astrocyte. E: Mitochondria from fish pseudobranch. F: Mitochondria from adrenal cortex (steroid-secreting cells). (From Reference 2, with permission.)

ing and optimizing their metabolism to the ever-changing conditions. The question about the number and shape of mitochondria in yeast, *Saccharomyces cerevisiae*, took a different turn when a series of serial sections were analyzed to reveal that in the extreme there may be only one mitochondrion per cell, existing as a highly branched, continuous structure. A single section alone will show what appear to be discrete mitochondria with spherical or elongated shapes [5,6]. (See Section 3.2 for further details.)

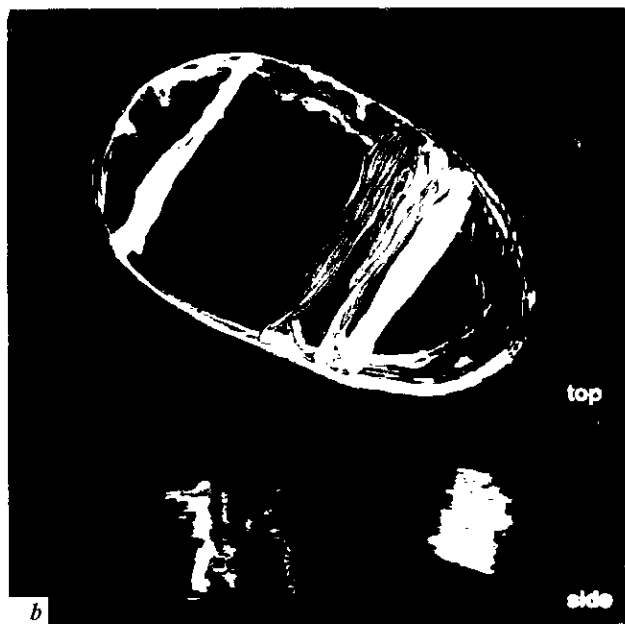
In multicellular organisms mitochondria are likely to have a more constant morphology in a given tissue, but different tissues have distinct energy needs. It may well be that an altered mitochondrial morphology is also related to functions of mitochondria not strictly linked to respiration. Steroid-secreting cells, such as Leydig cells, have mitochondria with an unusually wide range of sizes and often a very distinct array of tubular cristae [2]. Perhaps the most puzzling display of cristae morphology is found



*a*

sporadically in many tissues of diverse species. The cristae appear to be made of parallel, hexagonal arrays of tubules with a triangular cross section. This may be an extreme manifestation of cristae with sharp angulations regularly spaced along their length, which are found in mitochondria of cardiac cells, among others [2].

It is evident that the more subtle aspects of the variations in cristae morphology usually cannot be explained in terms of some sweeping generalization about surface area and respiratory activity. The inner mitochondrial membrane is an exceptional membrane with respect to the protein : lipid ratio. While in most membranes this ratio



**Figure 3.5** A: Mitochondria seen by electron microscope tomography. Cells were derived from chick CNS. B: The three-dimensional reconstruction was achieved after the volume was segmented manually, contouring into the regions bounded by the outer, inner, and cristal membranes. At the bottom of the figure individual lamellar sections of crista are shown, together with the tubular portions that connect the disks to the inner boundary membrane. (From Reference 3, with permission.) (See color plates.)

is close to 50:50, conducive to the image of “proteins floating in a sea of lipids,” the inner mitochondrial membrane has a ratio of approximately 75:25, suggestive of relatively densely packed proteins. Researchers focused on the inner mitochondrial membrane were among the most reluctant in their acceptance of the “fluid mosaic model” as a model for all biological membranes (J. Singer, personal communication). However, numerous studies have now provided strong experimental support for the view that the inner membrane is in a fluid state rather than a solid state [reviewed in References 7 and 8]. Thus, the proteins of this membrane cannot be exclusively responsible for its folding.

If the outer membrane has a fixed surface area and hence can confine the contents to a fixed volume, the inner membrane with a larger surface area must necessarily fold to fit into the available volume. Some order to this folding may be provided by the periodic attachment of the inner membrane to the outer membrane. Such rivetlike attachment points have been observed, and there is now excellent agreement that such attachment points may be made up of the components that are responsible for the import of peptides made in the cytosol. They may be formed transiently by the interaction of integral membrane proteins in the outer membrane with others in the inner membrane, thus aligning two channels through both membranes. Further details on such a proposed structure and its function in protein import will be presented in a later chapter.

Formation and maintenance of tubular cristae or cristae with a triangular cross section presents a greater challenge to the imagination. Theoretical studies on the conformation of membranes can lead to stimulating speculations [9], but experimental verification is still a major unachieved goal. The morphology of membranes such as the plasma membrane and the Golgi membranes is demonstrably determined by interactions with cytoskeletal elements, but there is no convincing evidence for the existence of such structures within mitochondria. Nevertheless, our view of the mitochondrial matrix as an amorphous soup of soluble enzymes is most likely too simple-minded.

In summary, the variation in mitochondrial morphology has been abundantly documented. Three-dimensional reconstructions from serial sections and more recently by confocal microscopy (even in live yeast cells) have removed some of the last ambiguities of interpretation of thin sections used in electron microscopy. A general correlation of the total area of the inner membrane (density of cristae) with the capacity for oxidative phosphorylation is apparent, but some of the more detailed aspects of cristae morphology in relation to function and activity are still relatively obscure. Even less is known about the control of this morphology by nuclear genes in the course of development and differentiation. Mitochondrial shapes and distributions within a cell may be controlled by extramitochondrial, cytoskeletal elements, but the establishment of the particular, cell-specific interior morphology remains mysterious, and therefore a challenge for the future.

## **3.2 INTEGRATION INTO THE CELL**

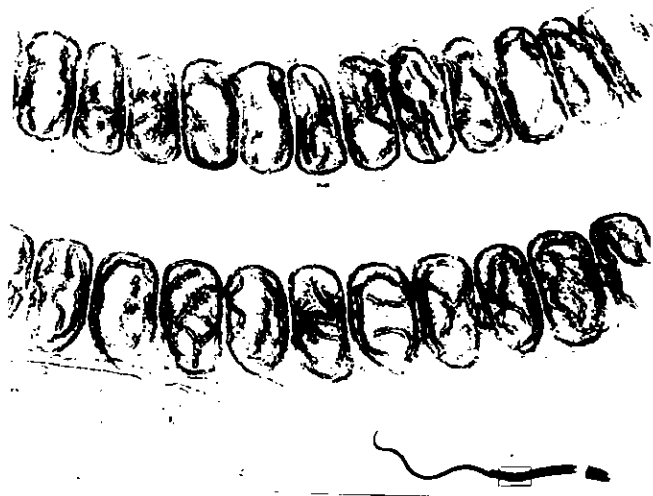
### **3.2.1 Distribution in the Cytosol**

So far in the discussion it has sufficed to consider mitochondria floating around in a cell, with highly diffusible molecules entering and exiting from the organelle. This view is certainly too simplistic, and there are now numerous observations indicating that mitochondria have a nonrandom distribution in cells; they can either be restrained in preferred positions, or move or be moved to sites where their output is needed. They are not only not static in their positions within a cell, but they are also quite changeable in their shape. Individual mitochondria can undergo shape changes, or shapes and sizes can be altered by processes of fusion or fission. Thus, some of the

dynamics may be due to internal or intrinsic driving forces. Other movements or dislocations are quite clearly due to interactions of mitochondria with the cytoskeleton and the possible involvement of molecular motors.

The studies on the dynamics of shape and position of mitochondria were begun early in this century before their identity had even been established. Stains were used, but the effect of these stains on the "viability" of these organelles was often not clear. In the 1950s the phase contrast microscope opened up new ways of exploring live cells and the visible subcellular structures whose identity was, however, not always fully established. When immunofluorescence techniques were perfected and specific antisera against various identified organelles and cytoskeletal elements became available, another significant expansion of opportunities for the study of mitochondria was achieved, but the need for permeabilization of cells also imposed a limitation: static images had to be interpreted, which could provide only hints of the dynamics in living cells. The first indications that mitochondria interact with the cytoskeleton were made in immunofluorescence studies with distinct labels for mitochondria and microtubules [10,11]. The most powerful technology became available in the 1980s when fluorescence microscopy and the discovery of new specific dyes with minimal harmful effects on mitochondria were combined to study mitochondrial distributions and dynamics in living cells. A landmark paper describing the laser dye rhodamine 123 was published by Chen's group in 1980 [12], illustrating the distribution of mitochondria in a variety of mammalian cell types. It also gave descriptions of alterations observed in cell transformation, and as a consequence of the disruption of microtubules by colchicine. Many studies followed, which have been expertly evaluated and summarized at various times [13–16]. The use of lipophilic cationic dyes to measure the membrane potential and the possibility of heterogeneity within a mitochondrial population of a cell with respect to staining, hence membrane potential, reflecting a difference in metabolic activity, is discussed in detail in Chapter 5. The present discussion deals with the distribution of mitochondria within cells and the mechanisms by which characteristic or functionally meaningful distributions are achieved or maintained. The most recent review by Bereiter-Hahn and Voth [16], in particular, includes many historical references and is comprehensive in its coverage of all aspects of the dynamics of mitochondria in living cells.

As in so many other areas of cell biology, the green fluorescent protein (GFP) has recently been used as a reporter to observe the dynamic behavior and fluctuating distribution of mitochondria in various cell types. When produced as a chimeric protein with a mitochondrial targeting sequence, it has been localized into the mitochondrial matrix of yeast cells [17] and HeLa cells [18]. Combined with powerful new imaging technology and computers, high-resolution three-dimensional images can be recorded almost continuously with live cells. The conclusions on the dynamic aspects of mitochondria are presented below. In a recent study of HeLa cells with differential labeling of mitochondria and the ER Rizzuto and colleagues [18] reported close appositions between mitochondria and the ER, and they estimated that about 5–20% of the mitochondrial surface was involved in such contacts. A possible significance is that at such sites microdomains are created where  $\text{Ca}^{2+}$  is released from stores in the ER mediated by  $\text{IP}_3$ . However, caution is in order, because both mitochondria and the



**Figure 3.6** Mitochondria aligned along an axoneme of a sperm tail. (Reference 2, with permission.)

ER network can become attached to microtubules by kinesin- or dynein-like motors, and thus their physical proximity may be coincidental. Furthermore, the density of these subcellular structures also makes apparent contacts quite likely.

It is clear that a single generic cell does not suffice to discuss the broad range of observations made on mitochondrial distributions in cells [reviewed in References 15 and 16]. Some general principles are emphasized here.

Mammalian cells in tissue culture (fibroblasts, cancer cells, etc.) have been popular for study because of ease of access, and important deductions have been made. When certain highly differentiated vertebrate or invertebrate cells are investigated, special considerations have to be included. Unique situations may be encountered in cells of lower eukaryotes, the kinetoplast of trypanosomes being one specific example. The discussion of events associated with cell divisions and the cell cycle usually ignores mitochondria, but it is obvious that there must be some mechanism assuring that each daughter cell obtains half the mitochondria of the dividing cell. This creates a special problem for the budding yeast *Saccharomyces cerevisiae*, to give one example, which is addressed below.

What dictates the distribution of mitochondria within a cell? In large and highly asymmetrical cells mitochondria may have to be localized in regions where the ATP

supply has to be high. Thus, mitochondria wrapped around or packed along the axonemes of sperm tails can be easily rationalized (Figure 3.6). In neurons, mitochondria have to be moved and distributed along the often considerable length of axons and into the growth cone. Skeletal and cardiac muscle cells require a very uniform distribution of mitochondria in the myofibrils along their entire length to provide a uniform supply of ATP to all the sarcomeres (Figure 3.7). A particularly impressive example of a highly regular arrangement of mitochondria in striated muscle cells is found in insect flight muscles. Rod cells in the retina appear to have their mitochondria concentrated in the cell body closest to the outer segment. In round yeast cells or in a gamete of *Chlamydomonas* the mitochondria are distributed around the cell periphery, perhaps to maximize their access to oxygen. Thus, it has been speculated that ATP (and/or ADP) gradients might be responsible for influencing mitochondrial distributions. Alternatively, the clustering of mitochondria within a cell might create gradients of other metabolites and ions such as calcium, and cause transient, highly localized perturbations, including pH gradients.

### 3.2.2. Interaction With Cytoskeleton

Mitochondria often tend to have an elongated, threadlike appearance, and when viewed in live cells there appears to be a rough alignment of their long axes. When they were colocalized with cytoskeletal structures, their association with microtubules became immediately apparent in several cell types. In fact, mitochondria sur-



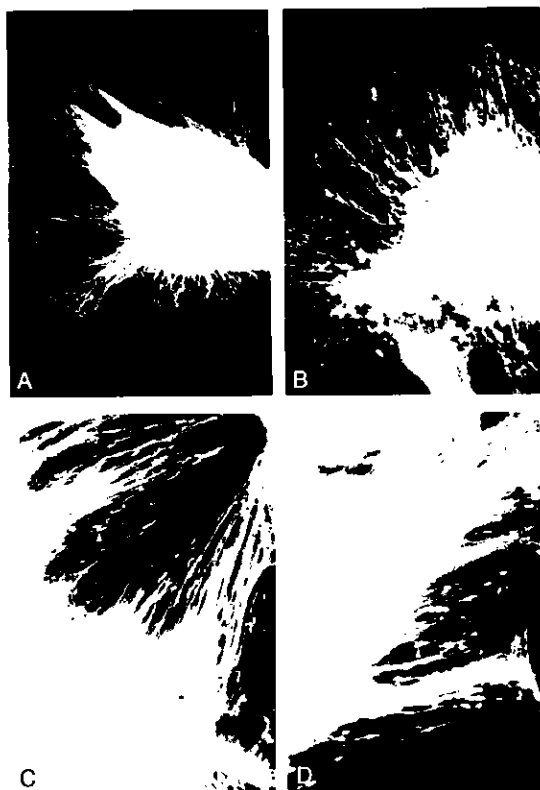


**Figure 3.7** A: Mitochondria distributed in skeletal muscle between sarcomeres. B: Cross section of cardiac muscle showing uniform distribution of mitochondria. (From Reference 2, with permission.)

rounding the meiotic spindle in insect cells were noted before the composition of the mitotic and meiotic spindles was known. Observations in the laboratories of Singer [10,11] and of Chen [12] provided definite proof, and many more observations in diverse cell types have followed these early studies (Figure 3.8).

One interpretation is that mitochondria are simply trapped and partially aligned within this cytoskeletal array of microtubules, and further dispersed by their own "intrinsic motility" (shape changes, fission and fusion, see below). At the other end of the spectrum of interpretations it has been argued that molecular motors attached to the outer membrane of mitochondria can become engaged with microtubules. In other words, dynein- and kinesin-like molecules have been looked for, initially based on the differential sensitivity of these motors to specific inhibitors. The evidence clearly points to an ATPase activity bridging the outer mitochondrial membrane and microtubules [16].

Before discussing the locomotion of mitochondria along cytoskeletal structures, it is relevant to consider whether the elongated shape of mitochondria may be related to



**Figure 3.8** Correlation of microtubule alignment and mitochondria. Antibodies against beef heart cytochrome oxidase and against chick tubulin were used in indirect immunofluorescence microscopy to localize microtubules and mitochondria in permeabilized cells. **A:** Normal rat kidney (NRK) cell stained with antitubulin antibody and rabbit anti-chick tubulin IgG. **B:** NRK cell stained with rabbit anticytochrome oxidase antibody and goat anti-rabbit IgG. **C** and **D:** NRK cells double-stained with antitubulin and anticytochrome oxidase antibodies. The axis of elongated mitochondria (arrowhead) is always parallel to the microtubule axis. (From Reference 10, with permission.)



or even dependent on their attachment to filamentous or long tubular structures, in the same way as the flattening of cells on a substrate is dependent on interactions between integral membrane proteins in the plasma membrane and extracellular matrix elements. In other words, is the sausage shape of mitochondria observed *in vivo* retained when mitochondria are isolated and highly purified *in vitro*? Unfortunately, the isolation of mitochondria tends to break up mitochondria, and the resulting mitochondrial preparations typically consist of smaller, spherical or only slightly elongated vesicles. A more gentle test of the dependence of mitochondrial morphology on intact microtubules can be made by treating intact cells with colchicine, and one of the earliest experiments of this type showed clearly that the distribution or orientation of mitochondria was perturbed, but their threadlike appearance was not altered by such a treatment. Therefore, some internal mechanism that controls the shape must be active. In the absence of an internal mitochondrial skeleton the answer has to be found in understanding the maintenance of the varying curvature of the membranes. Under some conditions, however, defined by specific mutations in extramitochondrial proteins, giant spherical mitochondria can be observed in yeast (see below).

Neurons, axons, and axonal transport have received the maximum of attention, since both retrograde and anterograde movement of mitochondria in axons has been observed by video-enhanced microscopy in intact axons as well as in extruded axoplasms. Closely related studies with systems of highly aligned microtubules of uniform polarity have been possible in unsheathed nutritive tubes of insect ovaria and in permeabilized reticulopodia of the giant amoeba *Reticulomyxa*. [The primary references for these studies are found in Reference 16]. A characteristic aspect of such movements is the saltatory nature of the displacements typical for motor-driven movements on cytoskeletal elements, and not compatible with simple Brownian motion. Unresolved problems are related to the bidirectional displacements, the specific speeds observed, the effectiveness (or lack of it) of known inhibitors such as vanadate (a dynein inhibitor in vertebrates), and the possible contribution of additional mechanisms, for example, an actin-based motility.

A recent series of reports from the laboratory of Hollenbeck [19–22] serve to illustrate the experimental approaches and the conclusions drawn from the observations. In one study chick sympathetic neurons were cultured in the continuous presence of either cytochalasin E to eliminate microfilaments (F-actin, MF), or in the presence of vinblastine or nocodazole to eliminate microtubules (MTs). Mitochondrial movements were followed by computer-aided video analysis after staining the organelle with rhodamine 123. In such neurons mitochondria continued to move with saltatory movements in both directions in neurites with MTs, but no MFs, but failed even to enter the neurites in the absence of MTs but presence of MFs. The dependence of mitochondrial movement on MTs was confirmed, and it appeared that MFs by themselves were insufficient to support the movement of mitochondria into the neurites. Interesting and partially contrasting results were obtained when the neurons were first cultured in the absence of any drug to allow for the normal development of axons. Subsequently the mature neurons were treated with either cytochalasin E or with vinblastine. The drugs disrupted the cytoskeletal structures as before, but in this case the axons already contained mitochondria. Now the average mitochondrial ve-

locity increased in both directions in axons lacking only MFs, but net directional transport decreased. Surprisingly, in axons with MFs, but no MTs, mitochondria also moved in both directions at a reduced average velocity and excursion length; net retrograde transport was favored under these conditions. When both drugs were added to eliminate both MTs and MFs, but leaving neurofilaments, no movement was observed. The studies clearly support an actin-based motility of mitochondria, and suggest that mitochondrial movement in neurites is a complex process involving several motors, each with characteristic intrinsic properties and presumably able to fine-tune mitochondrial distributions for purposes yet to be understood.

There have been numerous investigations aimed at establishing an interaction between mitochondria and intermediate filaments. A codistribution has been observed in a few instances [see Reference 16 for references], but since there are few systems with highly organized, regular arrays of such filaments, model systems, especially cell-free systems, are lacking. Some observations point to a role of desmin in anchoring mitochondria in skeletal muscle cells to preserve their relative positions during repeated contraction-relaxation cycles, but a more definite proof is lacking. In nonmuscle cells deliberate attempts to demonstrate an alignment of mitochondria with intermediate filaments have failed.

Further impetus to relate mitochondria and intermediate filaments has come from studies in Yaffe's laboratory [23] with the yeast *Saccharomyces cerevisiae*. Taking a genetic approach, this laboratory has isolated mutants defective in mitochondrial inheritance, that is, these mutants fail to transfer mitochondria into daughter buds. A gene for an essential protein was thus identified (*MDMI*), and the Mdm1 protein was found to have modest sequence similarity to animal intermediate filament proteins. In vitro this protein can form intermediate filamentlike (10 nm) structures, while in vivo immunochemical staining reveals a punctate distribution of the antigen. See Section 3.2.4 for further discussion.

### 3.2.3. Mitochondrial Shape Changes

The phenomenon referred to as "intrinsic mitochondrial motility" [16] has been observed in animal cells, in fungal hyphae, in algal cells and in the cytoplasm of plant cells where cytoplasmic streaming is absent to allow longer term observations. A mitochondrion appears to be able to slither, creep, or slide at rates ranging from 2 to 30  $\mu\text{m}/\text{min}$  in the direction of its long axis. The displacement is accompanied by subtle shape changes: slight thickening or contractions along the axis, possibly reflecting localized changes in the internal arrangement of the cristae. Microinjection studies with ATP and/or ADP have shown that these reagents can immobilize mitochondria, and, in general, the influence of metabolic conditions on mitochondrial motility have been interpreted in favor of intrinsic activities driving their displacements [16].

Intrinsic dynamic changes in mitochondria are most strongly indicated by the well-documented studies on mitochondrial fusions and fissions. Such events can be followed in the phase contrast microscope by time-lapse photography, and in some cases observations on live cells have been elegantly related to follow-up examinations with the electron microscope [16] (Figure 3.9).

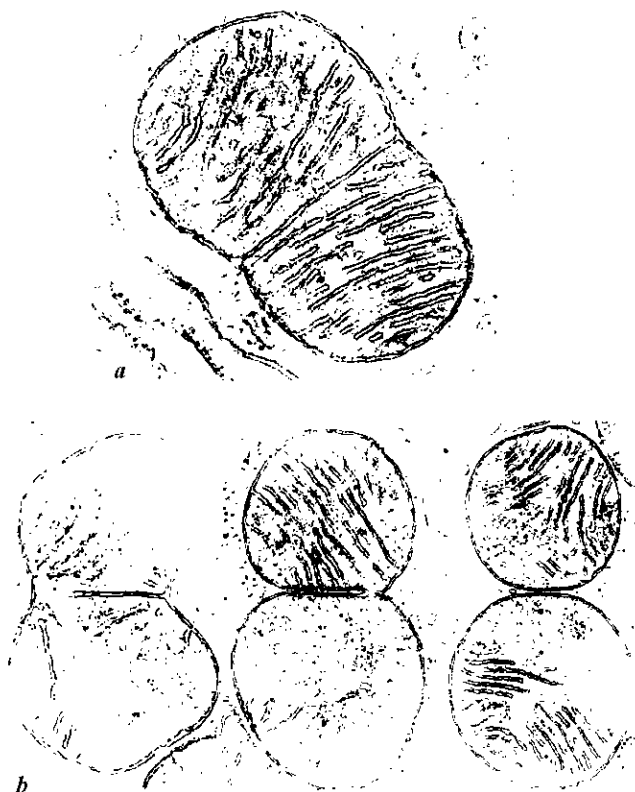


Figure 3.9 A: Dividing mitochondrion. B: Successive stages in a dividing mitochondrion. (From Reference 2, with permission.)

In particular, fission is the postulated mechanism for mitochondrial proliferation: the insertion of new components into the matrix and all the membranes will cause mitochondria to grow, and eventually there must be a signal for such a mitochondrion to divide, although insertion of lipids and proteins into the inner membrane may simply increase the number of cristae. Some spectacular electron micrographs [2] show-

ing bilaminar septa during progressive stages of mitochondrial divisions leave one with little doubt about the overall mechanism, but they raise new questions about the control of this process, which remain largely unanswered. Since an inner and an outer membrane are involved, the process is not a simple pinching-in-half of a membrane vesicle, and it may be more analogous to the division of a bacterium, where the plasma membrane and the cell wall represent separate entities surrounding the particle. Microscopic observations suggest that fission is preceded by stretching, but constrictions may appear at one site transiently and disappear without the initiation of fission [16].

There is an alternative view of the division of mitochondria, based on observations with a select number of organisms. In the unicellular red alga *Cyanidioschyzon mero-lae* a structure referred to as an MD ring (mitochondrion-dividing apparatus) was observed. This ringlike structure was found on the outside of mitochondria, and it was interpreted to function in a manner analogous to the contractile ring in cytokinesis [24]. T. Kuroiwa and colleagues [24] claimed that cytochalasin B inhibited "mitochondriokinesis," and deduced the presence of actin filaments in the ring. However, these filaments could not be stained with anti-actin antibodies or with phalloidin. Because such rings have not been observed in any other organism, the generality and significance of these observations must remain in doubt.

Collisions brought about by the motility of the organelles may lead to fusions, especially when at least one tip makes contact with another, or with the side. Contact sites and the creation of contact zones are inferred from observations with the electron microscope, but unfortunately such images represent a static situation that cannot be further resolved in time. The biochemical characteristics of such contact zones remain largely unexplored, although hexokinase and creatine kinase appear to be concentrated at such locations. Needless to say, fusion involves both the outer membrane and the inner membrane, presumably in a sequential fashion. Another complicated event following the fusion is the rearrangement of the cristae. The potential participation of mediators of fusion analogous to the SNARE protein assemblies in the secretory pathway is at this time a mostly speculative idea. In contrast to the secretory pathway, the membrane fusions occur between identical organelles, although some asymmetry between docking vesicle and target membrane may be transiently created when a mitochondrial tip encounters a side.

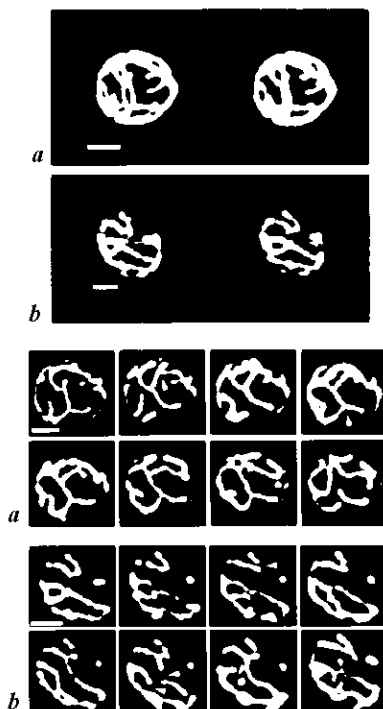
Mitochondrial fission appears a priori to be the more essential event, since mitochondria have to multiply and ultimately be divided between daughter cells. The rationale for the fusion of mitochondria in most normal cells is less obvious, unless it serves as a mechanism to scramble and "homogenize" mitochondrial genomes among the many mitochondria and to promote recombination. Fusion events involving mitochondria that are developmentally programmed have been characterized in many systems [25]. Section 3.2.5 details some salient insights into this phenomenon in the context of cell differentiation.

Questions raised with regard to the problem of mitochondrial shape, number, fusion, and fission are intimately related and illuminated in organisms where the number of mitochondria is small, and where even a single mitochondrion may exist in a

highly branched, reticulated shape, such that a section viewed in the electron microscope gives the appearance of multiple mitochondria. Serial sections and image reconstructions have revealed that in the yeast *Saccharomyces cerevisiae* a cell contains 1 to 10 such reticular structures expanded below the cortex of the cell [5,6]. A state of the art reexamination of this feature was recently presented by Nunnari and colleagues [17], who modified GFP for import into mitochondria to be able to use wide-field fluorescence microscopy for the acquisition of three-dimensional images over long time intervals. The use of GFP avoids the problems of photobleaching. Beautiful three-dimensional images were obtained, but more significantly, time-resolved images from single optical sections yielded quantitative data on fusion and fission events. While the authors are cautious to state that the resolution does not unambiguously allow the distinction between fusion and close juxtaposition, many events leading to stable connections were documented. During vegetative growth of wild-type cells  $0.39 \pm 0.14$  fission events per minute and  $0.40 \pm 0.08$  fusion events per minute were observed, indicative of a highly dynamic situation. The matching figures indicate a steady state in which a continuous network undergoes constant fragmentation and reassembly. They confirmed previous observations that at least one tip (end) was involved, where the putative fusion machinery may be located (Figure 3.10).

A similar study with GFP in HeLa mitochondria [18] also obtained high-resolution three-dimensional images at 30-second intervals, and it was concluded that these mitochondria also formed a connected, continuous, and highly dynamic network, exhibiting growth, retraction, and fusions to other portions of the network. Photobleaching of a small area within a cell was followed by a rapid recovery (5–20 minutes) of GFP fluorescence within that area.

A second important problem addressed by the studies of Nunnari and associates [17] is concerned with the behavior of the mitochondria and the mitochondrial genomes when two cells fuse, as in mating of yeast cells. This aspect will be considered in more detail below. The observation of constant fusions and fissions of mitochondria in a given somatic cell has implications for the geneticist. In the absence of additional information (see below) the dynamic behavior also leads to the conclusion that mitochondrial genomes in a heteroplasmic cell are continuously mixed and redistributed, with the result that a given mitochondrion contains both types of genomes at any time. Thus, in a heteroplasmic cell with a mitochondrial mutation in a tRNA gene, for example, protein synthesis should be compromised in all mitochondria. Can a population of mitochondria arise within a heteroplasmic cell that has a homogeneous population of mtDNAs? A theoretical answer to such a question depends on several factors. First, what are the proportions of the two types of genomes? Second, how many genomes are present on average in a single mitochondrion? Third, how frequent are fusions and fissions relative to mtDNA replication cycles? Finally, does a mitochondrion with one or the other genome in the majority (mutant or wild-type) have any selective advantage in the expanding mitochondrial population. This is different from an mtDNA with a large deletion having a replicative advantage. The above prediction could be seriously challenged when additional evidence for physical and biochemical segregation of mitochondria within a cell (e.g., during differentiation) can be obtained.



**Figure 3.10** Mitochondria in living yeast cells. Green fluorescent protein was modified to be imported into mitochondria. Wide-field fluorescence microscopy was used for the acquisition of three-dimensional images over long time intervals. **Top:** Three-dimensional images of yeast mitochondria in wild-type cells grown on galactose. Two different cells are shown in A and B. **Bottom:** Fission and fusion of mitochondria. Time-resolved images from a single optical section of  $0.2\ \mu\text{m}$ . Time intervals from left to right in 3-minute intervals. Two different cells are shown in A and B. (From [17], with permission)

### 3.2.4. Distribution During Cell Division

When a cell contains a large number of mitochondria more or less evenly distributed throughout the cytoplasm, and doubling in number and volume during the cell cycle, no significant conceptual problem exists for distributing these mitochondria evenly into the daughter cells during cytokinesis. Furthermore, if mitochondria become

attached evenly to the newly formed microtubules of the mitotic half-spindles, their segregation is easy to visualize. The problem during the cell cycle, therefore, is to ensure a doubling of mitochondrial mass and number, and to maintain a constant ratio of nuclear to mitochondrial genes. Cell differentiation during development, and mitochondrial proliferation and turnover in terminally differentiated cells like muscle and neurons pose a different problem.

A special problem arises when cell division occurs not by the classical process of cytokinesis in which a larger cell is partitioned into two cells either by a contractile ring as in animal cells, or by the assembly of a phragmoplast in plant cells. In the budding yeast *Saccharomyces cerevisiae* a bud is started and enlarged during the cell cycle, and all the constituents for a new viable cell have to be transported through a narrow connection between the bud and the mother cell. How this is achieved for the nuclear chromosomes has been a subject of study for some time, but the problem of equipping the bud with mitochondria has been illuminated only relatively recently by pioneering studies in the laboratory of M. Yaffe [expertly reviewed in Reference 23]. Taking a genetic approach, the Yaffe laboratory has identified yeast ts mutants which are defective in the transfer of mitochondria and nuclei into growing daughter buds at the nonpermissive temperature.

The original *mdm1-1* allele, and additional *mdm1* alleles isolated later, define a gene encoding a protein (Mdm1p) with several intriguing properties. The protein is essential for viability. With some mutant proteins the problem of mitochondrial distribution can be separated from nuclear distribution. The protein sequence has some similarity to intermediate filament proteins of vertebrates, and it forms 10-nm filaments in vitro. Filament formation fails at the nonpermissive temperature in vitro with the protein encoded by the original *mdm1-1* (ts) allele. On the other hand, indirect immunofluorescence microscopy with anti-Mdm1p antibodies reveals punctate staining distributed throughout the cytoplasm of wild-type cells. With mutant alleles affecting mitochondrial inheritance, the mitochondrial morphology in the mother cells is also abnormal. Instead of the usual reticulated, tubular wild-type structure, one finds small, round mitochondria in clusters. These observations invite speculation that a cytoplasmic filamentous network or scaffold exists that is responsible for the maintenance of the mitochondrial morphology of yeast discussed above, and that it also plays a major role in guiding presumably threadlike mitochondrial structures through the connection into the bud. It should be mentioned that fungi do not have the typical intermediate filament proteins of higher eukaryotes. The yeast filaments made from Mdm1p may represent an alternate solution to a problem that reached an evolutionary dead end, and they were replaced by proper intermediate filaments in more advanced organisms. The *mdm1* mutation does not appear to affect actin- and tubulin-based cytoskeletal functions.

Mdm1p may not be the only protein involved in this peculiar yeast cytoskeletal structure. Additional mutants isolated in the Yaffe laboratory have led to the characterization of other *MDM* genes, of which *MDM14* encodes a 35-kDa protein, which appears to interact with the Mdm1p, and is distributed in the cytoplasm in a similar fashion. Not unexpectedly, on second thought, some *mdm* mutants are affected in proteins in the outer mitochondrial membrane (*mdm10*, *mdm12*) [23,26]. Clearly, asso-

ciation with a cytoskeletal element implies the existence of a protein domain on the mitochondrial surface, which must at least bind to the scaffold, and perhaps even have motorlike properties. A related gene product in the outer membrane is the Mmm1p identified by Burgess and coworkers [27]. Depletion of these proteins has pronounced effects on mitochondrial morphology, shown for the *mdm10* mutant in Figure 3.11B, and null mutations yield a temperature-sensitive phenotype [28]. As pointed out by Yaffe [23], the fact that these null mutants grow at the permissive temperature on glucose (albeit slowly) suggests that a bypass or backup pathway must exist to assure mitochondrial transmission to daughter buds. Nevertheless, *mdm10* and *mdm12* mutants lose mitochondrial DNA and generate respiration-deficient cells at higher than normal rates.







Figure 3.11 A: Mitochondria in a budding, wild-type yeast cell. Note the movement of one elongated mitochondrion into the bud. B: Abnormal, giant spherical mitochondria in yeast cells with the *mdm10* mutation [28]. (Photographs provided by M. Yaffe.)

Least expected perhaps was the explanation for the failure of mitochondrial inheritance in the *mdm2* mutant. In this mutant the *OLE1* gene is affected, encoding a fatty acid desaturase [29]. Mitochondrial membrane fluidity evidently affects mitochondrial transmission, but it may be too simple-minded to think of a mitochondrion squeezing through a tiny pore connecting mother cell and bud.

Meiosis and sporulation in yeast represent a special kind of cell division in which most of the emphasis is usually placed on the behaviour of the chromosomes. What about mitochondria? One study has defined morphological transitions in the early stages followed by a dramatic fragmentation of a single large mitochondrion into

small spherical structures which were distributed to the spores [30]. Mechanisms for such events remain largely speculative at this time. If, as discussed above, fusion and fission occur normally at equal rates, one may simply have to inhibit fusions to promote the breakup of a large mitochondrion into smaller units. The phosphorylation or dephosphorylation of an essential docking protein may be all that is required, but identifying it is another problem.

Before meiosis can occur in yeast cells, one must have a diploid cell formed from the fusion of two haploid cells. When respiration-deficient yeast mutants were first investigated by means of mating experiments, and observations were made on the stability of phenotypes during mitotic divisions of the zygote, a puzzling observation was made. Zygotes containing heteroplasmic mtDNA populations generated homoplasmic progeny within approximately 20 generations, much faster than expected on the basis of stochastic models assuming random segregation [reviewed in References 31 and 32]. This observation is even more puzzling in light of the discussion about the dynamic nature of the large mitochondrial structure and the implied constant mixing of mtDNAs. A recent study by Nunnari and colleagues [17] has reinvestigated this problem with a most elegant and ingenious approach. Mitochondria of one haploid cell were labeled with GFP; mitochondrial proteins of the other mating type were labeled with the vital dye TMR-CH<sub>2</sub>Cl, which not only localizes in mitochondria because of the membrane potential, but eventually becomes covalently attached to mitochondrial proteins. When such cells were allowed to mate, the fusion and complete mixing of the two mitochondrial protein populations could be directly observed by wide field fluorescence microscopy; these observations were fully consistent with observations described above on the dynamic behavior of yeast mitochondria. The more provocative result was obtained when mtDNA from one mating partner was labeled selectively with bromodeoxyuridine (by a not totally trivial procedure). Its distribution in the zygote could subsequently be determined immunochemically after fixation and permeabilization of the cells. The results showed unequivocally that while mitochondrial proteins from the two mitochondrial populations mixed readily, the corresponding mtDNA populations remained segregated in the two lobes of the zygote derived from the haploid cells. This result was a visual confirmation of previous genetic experiments which had inferred the unequal distribution of mtDNA in the zygote from an analysis of mtDNA in zygotic progeny. In such progeny the type of mtDNA (genetically marked) depended on the position of the bud emergence from the zygote [33]. To interpret these findings, many questions remain to be answered about the nature of the attachment of the mtDNA either to structures within the matrix which restrict diffusion, or attachment sites which are connected to cytoskeletal structures on the outside.

As illustrated by the above discussion, genetic approaches are a powerful means of discovering genes in yeast encoding functions related to mitochondrial morphology, segregation, and stability. Mutants such as *mgm1* (mitochondrial genome maintenance [34,35]), and the series of *yme* mutants (yeast mitochondrial DNA escape [36]) represent additional examples which pose still major challenges for the future. No other organism is comparably advanced and genetically more tractable than yeast. However, while yeast has been the model system par excellence for understanding many funda-

mental aspects of the biology of mitochondria, other future problems related to cell differentiation and development may not have counterparts in yeast biology.

### 3.2.5 Behavior During Cell Differentiation

Developmentally regulated fusion of mitochondria has been described in a limited number of mammalian tissues, where its functional significance is obscure. It is observed in many lower eukaryotes [protists, fungi, algae; reviewed in Reference 25], and in insect spermatogenesis, with much emphasis on *Drosophila* [2,37,38]. Cytological and electron microscopic descriptions [e.g., 38] have documented dramatic morphological changes associated with spermatid differentiation after the completion of meiosis, and the behavior of mitochondria during this period has attracted special attention. All mitochondria first aggregate next to the haploid nucleus, and then appear to fuse into two giant mitochondria. These two giant mitochondria remain intimately associated in a spherical structure called a Nebenkern. The mitochondrial membranes form a series of concentric layers resembling an onion in cross section. When the axoneme is formed and flagellar growth proceeds, the two mitochondria separate from each other and elongate along the axoneme. The juxtaposition of mitochondria and axoneme along the long axis ensures an optimum transfer of ATP to the dynein motors of the axoneme to support flagellar movement. The hydrodynamics of the sperm tail motion is optimized by a decreasing thickness along its length, and this is achieved by a gradual decrease in mitochondrial size (diameter) in the sheath, while the diameter of the axoneme remains constant. The entire process has many facets for which molecular details remain to be unraveled.

The use of *Drosophila* as a model organism in genetics and developmental biology has led to the accumulation of a large collection of mutants. Among the male sterility mutants a mutant termed *fzo* (fuzzy onions) was recognized to have a defect in the mitochondrial fusions leading to Nebenkern formation. As a consequence, postmeiotic sperm differentiation is arrested. In a typical illustration of the power of the "reverse genetic approach" Hales and Fuller [39] have mapped, cloned, and sequenced the gene, derived a protein sequence, and from it predicted the existence of a transmembrane GTPase. This protein is present on spermatid mitochondria during the relevant period when fusion takes place, as determined by indirect immunofluorescence microscopy. Homologs of the gene have been detected in mammals, nematodes, and yeast, and they constitute a novel family of multidomain GTPases. The biological function of this protein is of special interest because Rab GTPases have been well characterized as the enzymes that control the assembly of the SNARE complex required for vesicle docking in the secretory pathway [40].

A comprehensive review under the provocative title *Sexuality of Mitochondria* by Kawano and colleagues [25] contains an expertly written account of mitochondrial fusions and recombination between mitochondrial genomes in the life cycle of the true slime mold *Physarum polycephalum*. In this organism mitochondrial fusions are not serving in morphological rearrangements as required in spermatogenesis. The authors emphasize recombination ("mitochondrial meiosis") and view the process in the broader context of the evolution and manifestations of sex. This view is reinforced by

the finding that only strains of *P. polycephalum* harboring a unique mitochondrial plasmid can display mitochondrial fusion (mF), and the mF-promoting plasmid may be representative of parasitic, selfish genes on plasmids, which have been proposed on theoretical grounds to have played a role in the early stages in the evolution of sex.

The review contains a broad introduction to mitochondrial fusion and the formation of giant mitochondria and reemphasizes the distinction between random fusions of mitochondria as observed in animal cells and periodic fusions as observed in protists, algae, and fungi, where it may have a much greater genetic significance. It is relevant that fungi such as *Saccharomyces cerevisiae* and *Physarum polycephalum* have mitochondrial nucleoids (referred to as mt-nuclei in Reference 25), and therefore it becomes an issue whether these mt-nuclei also “fuse.” From the discussion in an earlier section it appears that the mitochondrial genomes of two mating partners in a yeast mating remain segregated for some time after fusion, although recombination has indeed been observed in yeast. The true slime mold has a complicated life cycle during which programmed mitochondrial fusion occurs during two phases (details in Reference 25). A germinating haploid spore gives rise to myxamoeba which can fuse to form a zygote from which a plasmodium develops. During the earliest stages of plasmodium formation mitochondrial fusion and fusion of mt-nuclei also occurs in mF<sup>+</sup> strains. Another series of mF plasmid-dependent mitochondrial fusions occurs during sporulation, which can be induced in the mature plasmodium. The large amount of mtDNA in *P. polycephalum* mitochondria facilitates the observation of mt-nuclei by DAPI staining, and the process of sporulation can be synchronized by an illumination cycle. A detailed analysis of mtDNA replication and of mt-nuclei behavior thus becomes possible and its description can be found in the original papers by Kawano and colleagues. Many intricate details require attention for understanding the mating-type system of *P. polycephalum*, and the genetic control of mtDNA transmission. Kawano and colleagues take advantage of restriction enzyme polymorphisms (RFLPs) in the mtDNA of various strains to do mitochondrial genetics.

The Mif<sup>+</sup> and Mif<sup>-</sup> phenotypes with respect to mitochondrial fusion discovered first were ascribed to the existence of a single transmissible factor, which eventually was identified as a ~16 kb mitochondrial plasmid. Plasmid sequences can also be found integrated into the mtDNA, and even mif<sup>-</sup> strains were found to have certain integrated plasmid sequences. It was concluded that the missing sequences were responsible for the Mif<sup>+</sup> phenotype. A further analysis of the mitochondrial fusion frequencies in mif<sup>+</sup> strains also established the presence of nuclear alleles controlling the frequency of mitochondrial fusion, but their precise nature remains to be elucidated.

The linear mF plasmid has been sequenced. Ten potential ORFs have been identified, but deduced amino acid sequences do not show significant homology with any amino acid sequences in the SWISS-PROT database, except for one which has been tentatively identified as a DNA polymerase [41]. Closely related DNA polymerase sequences have been found on other linear mitochondrial plasmids.

Several important biological questions related to the mF plasmid can be raised, but answers are not available or incomplete. Some of these have been elaborated in the Section 4.1.6. To restate them briefly here:

1. The replication of a linear plasmid DNA poses special problems which can be overcome by the existence of terminal inverted repeat sequences (TIRS) and mechanisms for replication initiation and priming.
2. The nature of the gene products encoded by the plasmid is of interest to answer several questions.
3. How is the plasmid maintained in the mitochondria?
4. What functions are necessary to promote mitochondrial fusions?
5. How do plasmid sequences become integrated into mtDNA and what are the biological consequences of such a recombination?

In summary, the discussion of developmentally regulated mitochondrial fusions is not intended to be exhaustive, but examples have been chosen to illustrate the potential rationale for the evolution of mechanisms of fusion. It is conceivable that mitochondrial fusions were originally part of the overall process of sexual reproduction, including genetic recombination of all genomes in the organisms. In organisms such as mammals where recombination between mt genomes is no longer observed at any significant frequency, the rationale for fusion of mitochondria in fibroblasts is less obvious, unless one views it as the simple reverse of fission, where fission is required for mitochondrial multiplication. The cell-specific and tissue-specific regulated fusion seen in these organisms may represent special adaptations of the more primitive mechanisms observed in lower eukaryotes.

### 3.2.6. Turnover and Degradation

In Chapter 4.8 a review of our current understanding of regulated protein degradation in mitochondria is presented. The emphasis there is on selective protein degradation by intramitochondrial ATP-dependent proteases. In the present section only a brief summary of what is known about turnover of the entire organelle will be presented. In general, the bulk of mitochondrial proteins are quite stable over prolonged periods of time. In mammalian cells half-lives are measured in days, if not weeks. In yeast, a recent study found that mitochondria labeled with imported GFP (mito-GFP) did not become significantly altered over a period of 7 hours even when the yeast cells were shifted from aerobic conditions to anaerobic, glucose-repressed conditions.

Indirect evidence for some mechanism related to turnover can be deduced from the age-dependent onset of neuropathies and myopathies and the associated increase in the fraction of mutated mitochondrial genomes in subpopulations of muscle or neuronal cells. The time scale for such a change in the genetic and biochemical properties of mitochondrial populations is still not well defined, and may be very difficult to measure precisely *in vivo* over a period of years.

There are some physiological conditions where cells are forced to resort to extreme measures to stay alive. Under prolonged and severe starvation many cells initiate a process referred to as autophagy [42–44]. Subcellular organelles can then find themselves engulfed by lysosomes (in animal cells) or by the vacuole (in yeast and

plant cells), and this internal degradation is presumably initiated to mobilize resources for the most essential functions for survival.

### 3.2.7. Unsolved Problems for the Future

There are several broad areas in which more information is needed, and future progress can be expected. Such progress is likely to be more rapid in organisms in which genetic approaches are possible such that mitochondrial morphology and behavior can be manipulated or modulated. Yeast is the organism of choice here. It is anticipated that interesting new proteins and their genes will have homologues in other organisms, allowing the information to be generalized or extended in other systems. The fruit fly *Drosophila* may be a second experimental genetic system in which to study mitochondria in the context of development and differentiation of specialized cells. Other problems may be unique to vertebrates, for example, and even to specific cell types and tissues.

Among the major problems one could list the following:

1. What controls the total surface area and hence the elaboration of cristae in a mitochondrion in a given tissue? Why do the cristae have such unusual morphologies in some mitochondria?
2. Is the matrix of mitochondria organized in a way that controls the position of the mtDNA, the location of protein synthesis, and perhaps even the shape of mitochondria by means of internal structural elements?
3. What are the structures (protein domains) on the surface of mitochondria capable of interacting with diverse cytoskeletal elements via molecular motors? How do such interactions contribute to the changing shape of mitochondria, to their movement within the cell, and their accumulation in subcellular regions?
4. What forces control division and fusion of mitochondria? The fact that two membranes are involved adds complexity. What controls the specificity and the timing, with reference to cell division and differentiation?

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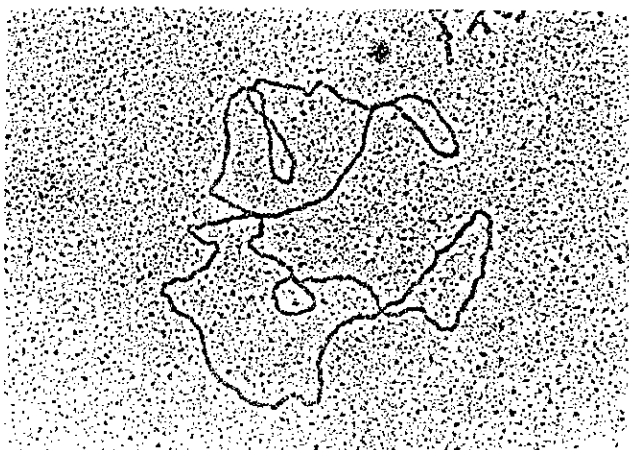
## BIOGENESIS

### 4.1 THE MITOCHONDRIAL GENOME

#### 4.1.1 Introduction

The discovery of DNA in a compartment other than the eukaryotic nucleus was truly a discovery with far-reaching possibilities and consequences. It not only shed new light on theories concerned with the origin of this organelle, and ultimately provided overwhelming support in favor of the endosymbiont hypothesis (see Chapter 2), but it also opened totally new vistas in the study of the biogenesis of mitochondria. At once the possibility was recognized that this subcellular structure was likely to contain genetic information required for its own assembly, and an immediate follow-up question was: How much? The earliest estimates came from studies with the electron microscope, after procedures were refined to isolate mtDNA molecules cleanly and in sufficient quantities [1–4] (Figure 4.1).

MtDNA molecules derived from diverse multicellular animals (metazoans) were found to be circular structures, and occasionally catenated dimers or higher oligomers, with contour lengths corresponding to ~16,000 nucleotides. It was obvious that only a limited number of genes could be present. During the past two decades dozens of mitochondrial genomes from animals, plants, and fungi have been characterized genetically and even sequenced [5]. A few milestones deserve special mention. The first complete mitochondrial genome to be sequenced from any organism was that of humans [6], and soon thereafter the bovine sequence became available [7]. Other than the genomes of bacteriophages and animal viruses, it was the first biologically well defined and distinct DNA molecule, one might even say chromosome, to become known in its entirety. Its completion with the technology of the day was a major achievement.



**Figure 4.1** Mitochondrial DNA from an oocyte of *Xenopus laevis*. (From Reference 4a, with permission.)

By contrast, the first complete mitochondrial genome from a plant (*Marchantia polymorpha*, liverwort) was not published until 1992 [8,9], and an even larger sequence from *Arabidopsis thaliana* (mustard) has appeared recently [10]. The latter is the first complete sequence from a flowering plant. The animal mtDNA was "only" 16,569 nucleotides long, while the plant mtDNAs contained 186,608 and 366,924 nucleotides, respectively, more than 20 times as many in the flowering plant compared to vertebrates. The following sections indicate that sizes of mitochondrial DNA vary considerably between metazoa, plants, and fungi, yet this difference is not necessarily paralleled by a proportionate difference in the number of genes retained by these genomes. The examples chosen will illustrate and support a number of generalizations, but at the same time they are selected to impress the reader with the tremendous variations found in different organisms. Clearly, one would like to understand the biological significance of any observed differences. One point of view is that the transfer of genes from the protomitochondria to the nucleus has progressed to different extents in different organisms purely by chance, and that further transfer of genes will occur in the future. Does the observation of a limited number of genes in mitochondria represent a snapshot in evolutionary time, or is it possible to interpret this finding in terms of structure and function? In metazoans the selection appears to have forced the reduction of the mt genome to its absolute minimum in terms of nucleotides, that is, the highest possible density of genes per unit length of DNA. It is not absolutely clear whether this represents a limit to the genes that can be transferred to the nucleus. At

one time it was possible to argue that rRNAs and tRNAs for translation must be transcribed from genes inside the mitochondria because researchers were unaware that the highly charged polynucleotides could be imported across two membranes. However, in the meantime, the import of tRNAs has been observed in plant mitochondria, in protozoa, and in yeasts. The proteins encoded by vertebrate mtDNA are very hydrophobic, prompting arguments that they also could not be imported from the cytosol. Such a rationalization fails to explain why the two integral membrane proteins of complex II are imported from the cytosol in most organisms. In plants and fungi there is similar solid evidence that a very extensive reduction in the number of genes has occurred, but at the same time many other sequences, mostly of no functional significance, have been acquired, either by transfer and scavenging from other sources, or by some poorly understood mechanism of amplification of intergenic sequences, or by multiplication of transposon-related elements. It is particularly noteworthy that many nonmetazoan mt genes have introns, and some of these introns contain ORFs.

When intergenic sequences are ignored, it becomes clear that the genetic information in the mitochondrial genome is remarkably similar in a majority of organisms. This could be because the loss of genes from the original symbiont was essentially complete before extensive branching of the phylogenetic tree occurred, or alternatively, that this limited number was achieved independently, leaving only those genes inside the mitochondria whose products could not be imported at all. As mentioned above, this argument is no longer sustainable for the tRNAs. At the same time, modern molecular genetic techniques have made it possible to knock out mitochondrial genes and complement them with appropriately engineered versions in the nucleus (codon usage, import signals). Such experiments argue against the absolute necessity of retaining certain genes in the mitochondria.

Although most of the organisms examined have circular mtDNAs, occasionally the discovery of linear mitochondrial DNAs is claimed. Initially dismissed as artifact, or as a very rare exception to the circular genomes expected from a prokaryotic ancestor, the number of such examples has grown over the past few decades. Diverse organisms from among the ciliata (for example, *Paramecium aurelia*), the algae (*Chlamydomonas reinhardtii*), the fungi (*Candida*, *Pichia*, and *Williopsis* species), and oomycetes are represented in a recent review of this subject [11]. An even more radical view gaining acceptance is that at least in plants and fungi (including *Saccharomyces cerevisiae*) the major form of mtDNA in vivo is linear [11]. The physical size of these linear mtDNAs is in the 30–60 kb range. Several criteria have been defined by investigators to distinguish true linear mtDNAs from linear concatomeric molecules arising from rolling circle mechanisms of DNA replication and other mechanisms. The most intriguing characteristic is a homogeneous terminal structure functionally analogous to telomeres. However, mitochondrial telomeres from diverse organisms do not share consensus sequences or sequence motifs indicative of a uniform solution to the problem of replicating 5' ends.

It has been argued that the existence of linear mtDNA does indicate an independent evolutionary origin, since even closely related fungi may have either linear or circular mtDNAs. Rather, the linear form may have arisen by accident, and become stabilized by the fortuitous existence of a replication machinery capable of dealing with linear ends [11].

### 4.1.2 The Mitochondrial Genome in Metazoans

The size range of mtDNAs found in multicellular animals is relatively narrow (~17 kb), with some exceptions varying from 14 kb in the nematode, *Caenorhabditis elegans* [12], to 42 kb in the scallop, *Placopecten magallanicus* [13], and all are single, circular DNAs. A curious exception is the finding of K. Warrior and J. Gall [12b] in 1985 that the mitochondrial genome of the cnidarian *Hydra attenuata* consists of two unique, linear DNA molecules of 8 kb. The exceptionally large scallop mtDNA may represent an extreme; it has not been sequenced, and may even consist of a duplication. Complete sequences are now available from various mammals, chicken (*Gallus domesticus*), toads (*Xenopus laevis*), the sea urchin (*Strongylocentrotus purpuratus*), the fruit fly (*Drosophila yakuba*), the nematode (*Caenorhabditis elegans*), and the sea anemone (*Metridium senile*). This list is not exhaustive, but it is striking that deviations in length from the published human sequence (16,569 kb) are generally less than 1 kb (see Table 4.1). A metazoan mtDNA database is being maintained by Dr. M. Attimonelli at the University

**TABLE 4.1 Sequenced Metazoan Mitochondrial Genomes**

| Class         | Organism                             | Genome Size (kb) |
|---------------|--------------------------------------|------------------|
| Nematoda      | <i>Ascaris suum</i>                  | 14,284           |
|               | <i>Caenorhabditis elegans</i>        | 13,794           |
|               | <i>Meloidogyne javanica</i>          | 20,500           |
| Insecta       | <i>Drosophila yakuba</i>             | 16,019           |
|               | <i>Anopheles quadrimaculatus</i>     | 15,455           |
|               | <i>Anopheles gambia</i>              | 15,363           |
|               | <i>Apis mellifera</i>                |                  |
| Crustacea     | <i>Artemia franciscana</i>           | 15,770           |
| Echinodermata | <i>Strongylocentrotus purpuratus</i> | 15,650           |
|               | <i>Paracentrotus lividus</i>         | 15,700           |
|               | <i>Asterina pectinifera</i>          | 16,260           |
|               | <i>Arbacia lixula</i>                | 15,722           |
| Cnidaria      | <i>Metridium senile</i>              | 17,443           |
| Amphibia      | <i>Xenopus laevis</i>                | 17,443           |
| Aves          | <i>Gallus domesticus</i>             | 16,775           |
| Osteichthyes  | <i>Crossostoma lacustre</i>          | 16,558           |
|               | <i>Ciprinus carpio</i>               | 16,364           |
| Mammalia      | <i>Bos taurus</i>                    | 16,338           |
|               | <i>Mus musculus</i>                  | 16,303           |
|               | <i>Rattus norvegicus</i>             | 16,298           |
|               | <i>Balaenoptera physalus</i>         | 16,398           |
|               | <i>Balaenoptera musculus</i>         | 16,402           |
|               | <i>Phoca vitulina</i>                | 16,826           |
|               | <i>Halichoerus grypus</i>            | 16,797           |
|               | <i>Equus caballus</i>                | 16,660           |
|               | <i>Didelphis virginiana</i>          | 17,084           |
|               | <i>Homo sapiens</i>                  | 16,569           |

Data from Metazoan mtDNA Database (MmtDB; M. Attimonelli, University of Bari, Italy; see reference 5 for further details).

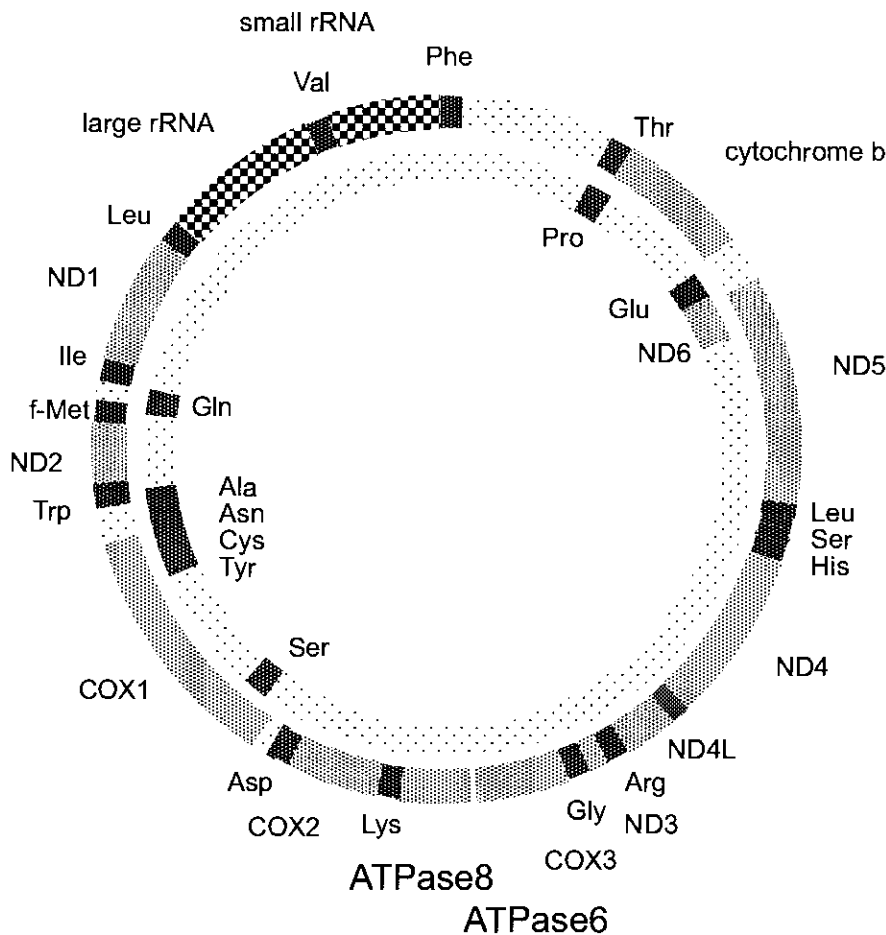
of Bari, Italy [5]. Updates on variations in human mitochondrial genomes can be found on the Internet (<http://www.gen.emory.edu/mitomap.html>) [14].

When the human mtDNA sequence was first published in 1981, not all genes were immediately identifiable, but a complete characterization of the remaining URFs (unidentified reading frames) was achieved in 1986 [15,16]. All the other metazoan mtDNAs were subsequently found to contain the same genes, with very few exceptions. On the other hand, the order of the genes is not always the same, suggesting that some rearrangements have occurred.

Metazoan mtDNAs encode two ribosomal RNAs (*s*-rRNA, *l*-rRNA), 22 tRNAs (with exceptions), and 13 peptides which become constituents (ND1-6, ND4L) of complex I (NADH-coenzyme Q oxidoreductase), complex III (cyt b), cytochrome oxidase (COI, COII, COIII of complex IV), and ATP synthase or complex V (ATPase 6, 8). The structure and function of these complexes in electron transport and oxidative phosphorylation are covered in detail in Chapter 5. It is relevant to describe here that each complex consists of multiple peptides, and among those one can distinguish peptides deeply embedded in the membrane (integral membrane proteins, IMPs), and others which are firmly associated with domains of the IMPs extending into the mitochondrial matrix. The IMPs contain a large proportion of very hydrophobic amino acids, and many have multiple membrane spanning segments based on hydropathy plots. It is often argued that such very hydrophobic peptides cannot be imported into the mitochondria, and thus their genes have been retained inside the mitochondrial matrix. Following, or even during, translation in the matrix the peptides can be directly inserted into the inner membrane.

With the set of 13 peptides identified in many diverse species, the identification of open reading frames in new mtDNA sequences from additional organisms can be accomplished relatively easily from sequence alignments and hydropathy profiles. It should be noted, however, that DNA sequences for individual peptides from different organisms, even closely related species such as mammals, have diverged quite significantly to the point where probes from one species cannot generally be used to detect DNA or transcripts from another. The extraordinarily high rate of base changes in vertebrate mtDNA will be the subject of a separate discussion (Chapter 7). And even though most metazoan mtDNAs encode 13 peptides, the size of the corresponding reading frame can vary considerably. For example, the ND6 peptide in mouse has 172 amino acids, while the same peptide in *C. elegans* has 145. The ATPase6 peptide has 226 residues in the mouse and only 199 in *C. elegans*. Other peptides, for example the cyt b peptide of complex III, or the peptides of cytochrome oxidase, exhibit less length variation from species to species. A more extensive compilation of such data can be found in the review by Wolstenholme [4].

The genetic information on the mt genome in metazoans is highly compacted on the circular mtDNA. The human mtDNA will serve as an example (Figure 4.2) to illustrate some of the general principles of organization of genes, but it should be stressed that there is variability, and some particularly striking exceptions will be noted to alert the reader of the deviations that can be expected in other organisms. Most, but not all, open reading frames for the peptides and the rRNA genes are separated by one or more tRNA genes, with few if any extra nucleotides in between.



**Figure 4.2** Schematic representation of the human mitochondrial genome map. The outer circle represents the heavy strand, encoding most of the peptides, the two rRNAs, and a large number of the tRNAs. ND1–ND6 genes encode peptides of complex I; COX genes encode peptides of complex IV.

Small-rRNA and large-rRNA genes are separated by a single tRNA in vertebrates and *Drosophila*, but by multiple tRNA genes in other invertebrates. It is interesting to be reminded that the dispersion of tRNA genes around the mt genome was already discovered before the arrival of cloning techniques. In 1976, by covalently labeling 4S RNA (tRNA) or rRNA with ferritin and hybridizing such “probes” to mtDNA, it was possible to map the adjacent 16S and 12S rRNA genes and 19 tRNA genes distributed around the circular DNA [17]. The transcription of the genome occurs in both directions, and hence open reading frames can be on either strand, although they are usually quite unevenly distributed between the heavy and light strands of mtDNA. (The

distinction is based on an asymmetrical distribution of bases, giving rise to a difference in density on alkaline CsCl gradients). Transcription, as will be discussed in more detail in Section 4.4, yields polycistronic RNA molecules. When the interspersed tRNAs are spliced out, individual mRNAs or rRNAs result. The mRNAs have extremely short or nonexistent 5' and 3' untranslated regions, and in some instances the first A from the polyadenylation step completes the terminal stop codon. Special problems are created for the initiation of translation which will be elaborated in a later section. In HeLa cell and other vertebrate mitochondria the ATPase8 and ATPase6 and also the ND4L and ND4 reading frames overlap by a few nucleotides without a separating tRNA. The mature mRNA is bicistronic, and translation initiation must occur internally. A detailed consideration of mechanisms will be deferred, but in the current context these observations emphasize the extreme economy of packaging genetic information into a small genome. A discussion of plant and fungal mtDNA will present a distinct contrast.

There is one region in metazoan mtDNAs that deserves special mention here. Flanked by two tRNA genes (tRNA<sup>pro</sup> and tRNA<sup>phe</sup> in vertebrates), a noncoding region of variable length has been found. It is 1122 nt long in humans, and 879 nt long in the mouse, and even larger in *Xenopus leavis*. Its sequence and function will be discussed in more detail under the appropriate headings (Sections 4.3 and 4.4). Here it suffices to state that this noncoding region contains a replication origin and the promoter regions for transcription in opposite directions. For these reasons it is being referred to as the *control region*. In spite of the functional identity, the control region is not very well conserved between species, and is polymorphic even within a single species such as humans. That is to say, there are short, functionally significant and conserved segments within the control region, while the remainder of the sequence is variable.

There are exceptions to the gene content and organization described for metazoans so far. They will not be catalogued exhaustively here, but a few examples will be presented to alert the reader to the possibilities that might be encountered with as yet unknown mt genomes. The genes for the rRNAs are generally encoded by the same DNA strand, separated by one or more tRNAs, but in the sea star they are encoded in opposite strands. This possibility should be considered when the analysis of a new mtDNA sequence fails to reveal one of the two rRNAs arranged in tandem. The creation of such a novel arrangement clearly requires an inversion of a portion of the mtDNA molecule. Other inversions and rearrangements must be responsible for the fact that the gene content is generally conserved in metazoans, while the gene order is conserved in fish, amphibians, and mammals, but not in birds, and even less so in invertebrates. Since processing of the polycistronic transcripts yields individual tRNAs, mRNAs, and rRNAs, the gene order may not be a matter of concern. Gene order is, however, of interest to the evolutionary biologists in their attempts to trace lineages over time.

As examples of variations in structural genes one can mention the following. In nematodes the ATPase8 gene is missing. In the sea anemone *Metridium senile* the COI and the ND5 genes contain a group I intron, and one of these (the COI intron) has an open reading frame potentially capable of encoding either an RNA splicase or an endonuclease. The ND1 and ND3 genes are contained within the intron of the ND5 gene.

Most surprisingly, the sea anemone mtDNA has only two tRNA genes, for tryptophan and formylmethionine. Since protein synthesis would obviously be impossible with only those two tRNAs, one has to confront the problem of how to import tRNAs into these mitochondria. Clearly, the import of such highly charged polynucleotides across two mitochondrial membranes presents a special challenge (see Section 4.7).

#### 4.1.3 Mitochondrial Genome in Plants

The mitochondrial genome in higher plants has many characteristics that probably make it one of the most interesting genomes to the molecular biologist [18]. It has a potentially large coding capacity, is constantly reorganized by recombination, has captured genes encoded in the nucleus in other organisms, suffers mutations that can affect growth and sterility, yields transcripts that have to be edited, or trans-spliced, and yet it lacks a subset of tRNAs that have to be imported from the cytoplasm. As can be expected, not all of these aspects are found all the time, and the functional-biological consequences of mitochondrial gene variations are discussed in Sections 4.1.3 and 7.6

It was mentioned earlier in this chapter that the first complete plant mtDNAs to be completely sequenced were those from *Marchantia polymorpha* [8] and *Arabidopsis thaliana* [10]. Sufficient information is therefore available now to permit several generalizations (see References 9, 19, and 20 for critical reviews and exhaustive listings of primary references). Most of the data were derived from flowering plants (Angiospermophyta), only 1 of 10 phyla within the plant kingdom. For this still narrow representation of plants the following statements can be made.

1. The mitochondrial genome is much larger than that in metazoans, ranging from 200 to 2400 kb. It can be noted that 2400 kb is within the range of some prokaryotic genomes. Original estimates were based on renaturation kinetics ( $C_0t$  curve analysis), others on summation of restriction fragments. Examinations by the electron microscope were inconclusive: heterodisperse linear and circular molecules were seen, and the possibility of breakage could not be discounted. Similarly, pulsed field electrophoresis of either whole or digested molecules gave results that were either confusing or inconsistent with data derived by other approaches. Large circular DNA molecules are known to remain trapped in the well of the gel. Another possible source of confusion is the presence of small linear and circular DNA molecules in the mitochondria of several plant species which appear to be mitochondrial plasmids which can replicate autonomously, and have open reading frames. One such plasmid, in all maize lines examined, carries the only functional tRNA<sup>trp</sup> gene [21]. A consensus view from restriction mapping and other approaches (cosmid walking) is that the mitochondrial genome can be described as a large "master circle" containing all the mapped sequences. Actual structures found are derived from this master circle by mechanisms described next.

2. The arrangement of genes on these large genomes is extremely variable, even between closely related genera. This fluidity is evidenced by altered restriction maps, and absence of conserved linkages between genes. As an explanation for this obser-



vation it has been proposed that plant mitochondria have an active recombination system [22,23], and in order to account for the structural rearrangements resulting from homologous recombination, the presence and participation of repeated sequences in both direct or inverted orientation has been noted. Repeats on the order of 1–10 kb have been found in the majority of plant mtDNAs, and a role for a larger number of small repeats in the larger mt genomes has been proposed to make recombination responsible for the scrambling of plant mtDNA sequences [9,24,25]. In considering plausible models, one has to remember that these genomes are circular, that the repeats may be direct or inverted, and that the distance between the repeats is also variable. A simple “master” circle with two direct repeats at some distance from each other will yield two subgenomic circles if recombination is intramolecular, and if recombination is relatively active, one expects to find a dynamic equilibrium between the master circle and the two subgenomic circles, a situation which has been observed in plants such as *Brassica* sp. which have the smallest mtDNAs. Clearly, recombination between the master circle and one of the subgenomic circles can give rise to larger circles with duplications. If multiple small direct and inverted repeats are present, the possibilities are too numerous to count. A more stringent definition of “recombination repeats” was proposed by D.B. Stern in 1994. In the simplest case, such repeats occur in two copies relative to other sequences in the genome, but a probe directed against the repeats may detect four distinct restriction fragments, reflecting four different genomic environments for the repeat which result from recombinational events. If more repeats are present, more such genomic environments are predicted and have in fact been found in petunia line 3704 and maize CMS-T [19].

On the other hand, not all repeats found are found in multiple environments, that is, there are repeats which appear not to have been involved in recombination. It should be emphasized that the postulated recombination has not been formally demonstrated, but the consistency of observations makes recombination the mechanism of choice for restructuring these genomes. Specifically, it is not yet clear whether recombination is an active process at the present, responsible for the polymorphisms detected by restriction mapping, or for the different cosmid clones in a library. An alternative explanation is that recombination occurred in the evolutionary past, but the molecules generated then are replicated and continuously maintained in the mtDNA population of a given species. Evidence in favor of an active recombination system may be found from observations made in the production of somatic cell hybrids by protoplast fusion and the subsequent generation of a hybrid plant. In a few cases studied, the protoplast parents had distinct mt genomes based on restriction analysis, and hybrid plants were found to be homoplasmic (have a genetically stable mt genome) with recombinant mtDNA molecules [19]. Curiously, recombination between mt genomes in somatic plant cell hybrids occurred at loci that were not normally considered to be recombination substrates [19,26]. A contrasting situation in mammalian somatic cell hybrids is discussed in Chapter 7.

In several cases known genes flank or even extend into the recombination repeat. This allows genes to occur in more than one genomic context, that is, to have potentially more than a single promoter. Whether this has any biological and hence selective function remains to be seen.

Among the genes found to be associated with repeats in some plants are various *rrn* genes, the *atp6* gene and the *coxII* gene, but no evidence for the presence of recombinase genes within such repeats has been found.

Recombination repeats, and hence recombination involving repeats are not essential, since at least one plant mt genome (*Brassica hirta*) has been found to be devoid of repeats. At the same time, recombination involving sites that are not repeats have been observed, both during tissue culture (see above) and in plants cultivated normally. In nature, such recombination events are rare, and they give rise to recombinant DNA molecules present at very low abundance relative to the majority mtDNA. Rare mtDNA molecules found at these substoichiometric levels have been termed "sublimons."

3. A third characteristic distinguishing the mitochondrial genes of higher plants from those of animals is that sequence divergence is strikingly slow. The plant mitochondrial genome is the most slowly evolving cellular genome so far characterized [9,27]. Thus, while large chunks of plant mitochondrial DNA have been moved around in multiple ways during evolution, sequences of individual genes have remained remarkably constant. The implication is that either mtDNA replication is exceptionally error-free, or that plant mitochondria have a very efficient mismatch repair system.

In view of the widespread use of *Arabidopsis thaliana* as the model plant species to study plant molecular genetics and development, an analysis of the mt genome of this plant will serve here to illustrate and support generalizations, and again, prominent or instructive exceptions will be noted at the end of this section.

With 366,924 base pairs the *A. thaliana* mt genome is at a medium position in the range found for plants. In light of its size, the gene content is of obvious interest. Because of previous results with related studies in plants it was not a total surprise to find only 57 genes on this genome, including three rRNA genes, 22 tRNA genes, and the "standard" set of genes for the mitochondrial respiratory chain complexes. For someone exclusively focused on animal mtDNAs up to now it will be news that plant mtDNAs generally contain genes for ribosomal proteins, and a 5S rRNA gene in addition to the rRNA genes for the small and large ribosomal subunits. A relatively unique set of genes found only in the mustard and the liverwort so far are five genes encoding proteins required for cytochrome c biogenesis (*ccb*). Three ORFs have not yet been identified from comparisons or homologies with genes in other organisms (plants or bacteria).

Among the "standard genes" for complexes I–V some contrasting observations between plants and animals can be made. In the mustard, liverwort, and a green alga, *Prototheca wickerhami*, there are two additional mitochondrial genes for complex I (*nad7* and *nad9*); plant mtDNAs encode three complex V genes (*atp1*, *atp6*, *atp9*), compared to two in human mtDNA (*atp6*, *atp8*), and in the liverwort but not the mustard the genes for the two membrane anchor proteins of complex II (succinate dehydrogenase) are found on mtDNA. The comparison of mustard, liverwort, and green alga suggests that plant mtDNAs encode ribosomal proteins, but their number may be different in different plants. Genes are encoded by both strands with no extreme strand bias.

Although the finding of 22 tRNAs may not raise any eyebrows at first, a more detailed examination has established that these 22 tRNAs are not capable of decoding all the codons found in the ORFs and that tRNA genes for six amino acids are missing. Previous results from the liverwort and the potato had alerted the field that the import of select tRNAs into plant mitochondria is required. Again, it is not possible to give a fixed number for plants, because it varies from 2 in the liverwort to 11 in the potato.

The summation of the sizes of the identified genes in *A. thaliana* mtDNA leaves a large amount of sequence unaccounted for. There are 23 identified introns; 18 of those are cis-splicing and comprise 30,764 nucleotides or 8.4% of the genome. Their size ranges from 485 nucleotides to 4000 nucleotides. Five trans-splicing introns cover at least another 4500 nucleotides of the genome. One group II intron appears to contain an ORF which may encode a maturase gene. Contrasting these data with those from the liverwort, one finds a total of 8 ORFs in type II introns (out of a total of 25) and 2 ORFs in type I introns (out of a total of 7). The intron locations are different between the two plants, and this is likely to be another highly variable situation differentiating higher flowering plants from lower plants, and possibly even flowering plants from each other.

The availability of several cDNA clones for protein genes in *Arabidopsis* mitochondria provides evidence for RNA editing, but the analyses are incomplete to verify all editing sites precisely.

There are numerous regions where short fragments from the chloroplast DNA have been integrated, amounting to approximately 1% of the mt genome. These fragments (30–930 nt) are unlikely to be functional, and the mechanism of their transfer from one organelle to the other is still obscure. Similarly, about 4% of the mt genome is of nuclear origin and identifiable as fragments of retrotransposons.

A general feature of most plant mtDNAs, the presence of repeats involved in recombination, was also found in the *A. thaliana* mtDNA, and from previous physical mapping and cosmid clones there is evidence for circular molecules of 233 kb and 134 kb which represent subgenomic circles arising from recombination. The finding and localization of two large repeated sequences of 6.5 kb and 4.2 kb is fully consistent with their participation in recombination to generate the smaller circles. The repeats have sequences conserved over their entire lengths. One of them extends into the *atp6* gene; 144 duplications of repeats in the 30 to 560 nucleotide size range were found, contributing 14 kb to the genome. Repeats are >90% identical, and are not implicated in any recombinational events so far.

Finally, 62% of the *Arabidopsis* mt genome is comprised of DNA sequences with no obvious informational content. In contrast to what is found in fungal mtDNAs, this spacer DNA exhibits no particular nucleotide bias, and its origin and biological relevance are completely obscure at this time.

#### 4.1.4 Mitochondrial Genome in Fungi

An extensive compilation of data for different fungi is compiled in an authoritative review by Clark-Walker [28]. The reader is referred to this article for details on many individual representatives of this large group of organisms. At this point one should not be surprised to find again significant variability, but there are also some features

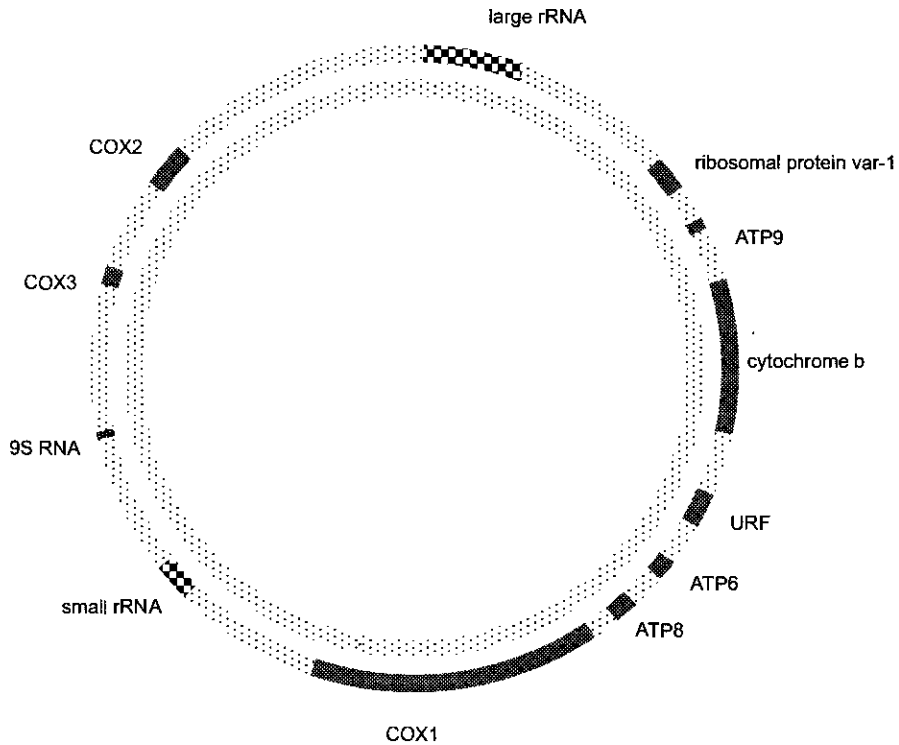
which distinguish this group from the metazoa and higher plants. The size of the mitochondrial genomes is typically between 40 and 60 kb, with extremes of ~20 kb for *Schizosaccharomyces pombe* (an ascomycetous fission yeast), and ~170 kb for *Agaricus bitorquis* (a filamentous basidiomycete). The average reader will be familiar with *Asperigillus nidulans* (33–40 kb), *Neurospora crassa* (60–73 kb), *Saccharomyces cerevisiae* (68–85 kb), *Ustilago cynodontis* (a rust) (76.5 kb), and others. The size of these genomes is on average three to four times larger than that of animals, but significantly smaller than that of plants.

The evolution and likely polyphyletic origins of fungi have been described and interpreted from morphological and biochemical studies, and a detailed analysis of their mt genomes, their sequence organization, and nucleotide sequence comparisons of individual genes such as rRNA genes can shed much additional light on this subject and confirm or refine the evolutionary relationships. The intent here, however, is to focus on common characteristics, and on novel aspects of genome organization and fluidity unique to fungi. These organisms form a large group with many taxonomic groups and subgroups, but at this level they must be lumped together to avoid drowning the reader in molecular taxonomy. The budding yeast *S. cerevisiae* mtDNA will serve as a useful reference point, since this organism has also become one of the best known model systems [29–31].

Among the expected genes present are the genes for the small and large ribosomal subunits, 24 tRNAs, cytochrome b in complex III, and three subunits of complex V (atp6, atp8, atp9). Notably absent are the genes (nad) for complex I, and it appears that *S. cerevisiae* mitochondria do not even have a typical complex I with the extraordinary number of ~40 subunits, that is found in most other organisms. On the other hand, the lack of nad genes is not characteristic of all fungi. A single ribosomal protein encoded by a gene called *VARI* is present, and there are several unidentified or open reading frames whose significance is still unclear. Strains of *S. cerevisiae* exist in which one or the other of these ORFs have been deleted, without any effect on growth on nonfermentable carbon sources, and they may not be expressed at all. There are also “optional introns” in *S. cerevisiae* mtDNA within which ORFs have been found. Examples are the *COXI* and the *CYB* genes, and it appears that the product encoded by these ORFs functions in the production of mature mRNAs by intron excision and splicing. In other words, the intron encodes an activity for its own removal, but when the intron is absent, the activity is not required for anything else. Optional introns account for a substantial part of the size variations in mtDNAs seen even within a single strain of yeast, for example, lengths of 68–85 kb found in *S. cerevisiae* (Figure 4.3).

The existence of introns, and the existence of “optional” introns raises several questions. The mechanism for removal of introns from RNA transcripts is appropriately discussed in the chapter on transcription and RNA processing in mitochondria. The acquisition and mobility of introns within the genome is a subject for the present context (reviewed in Reference 32).

*S. cerevisiae* strains exist which differ in the presence or absence of a 1132-bp group I intron in the large rRNA gene at the so-called omega ( $\omega^+$ ) locus of mtDNA. The  $\omega$  genetic system was discovered independently of the discovery of introns, but pioneering studies very rapidly related it to introns, and understanding its behavior



**Figure 4.3** Schematic representation of the yeast mitochondrial genome map. The tRNA genes are not shown; only the major genes for peptides and ribosomal RNA are indicated. Genes are separated by large stretches of AT-rich DNA, which are not drawn to scale here.

has been a major guide in the understanding of group I intron mobility in general. When crosses between  $\omega^+$  and  $\omega^-$  strains are made, the intron can move into the genome in which it was previously absent ( $\omega^-$ ), since in the progeny mt genomes with this intron are preferentially recovered. There is evidence that the intron has an ORF (235 nt) encoding the  $\omega$  transposase later characterized as a site-specific, double-stranded endonuclease, which is targeted to a specific sequence within the intron-free allele. It is speculated that site-specific cleavage initiates DNA recombination and conversion (reviewed in References 33 and 34). The  $\omega^+$  intron in yeast was initially considered a rare oddity, but eventually similar introns were found in other organisms and at other loci in the yeast mt genome [32]. The  $\alpha 14\alpha$  intron in the *COXI* gene can serve as another example [35,36]. It emerged that each intron-associated endonuclease was unique, with a different recognition site sufficiently complex to make it relatively infrequent in the genome. A plausible hypothesis proposed by Lambowitz [32] is that introns of group I (and group II) may initially have been self-splicing, with the involvement of a specific secondary structure. They can function only when inserted at sites where flanking exon sequences are compatible with splicing. ORFs were ac-

quired later in evolution and adapted as endonucleases to facilitate transposition, or as maturases which assist in splicing by stabilizing the RNA in the catalytically active secondary structure. Endonuclease and maturase functions may even be combined in a single protein as suggested for the  $\alpha 14\alpha$  intron.

The mechanism may be different for group II introns, and here speculation is driven by the finding that such introns may have ORFs encoding a protein related to retroviral reverse transcriptases. Mobility of the intron is therefore related to the RNA splicing reaction, where the RNA excision product could be reverse-transcribed to yield DNA which in turn is postulated to recombine with the intron-less gene [37]. Alternative mechanisms proposed include a reversal of the splicing reaction into a compatible location of a different RNA, or even integration into a single stranded DNA at a replication fork [32].

If introns can be mobilized during intra-species crosses, the problem of whether and how introns may spread between species is still in the realm of speculation, fired by observations that some introns in different species show higher sequence similarity than the flanking sequences. As in all discussions about intron evolution, one may have to distinguish between early and late introns and establish clear phylogenetic relationships before horizontal intron transmission can be proved.

A second source of interspecific and intergeneric size variation has been determined from sequencing the regions between genes of several mt genomes of fungi including *S. cerevisiae* [29–31]. They account for >60% of the *S. cerevisiae* genome and consist of sequences extremely rich in A + T (>90%), but there are also ~200 clusters of 20–50 bp which are rich in G + C. Circumstantial evidence (e.g., two nucleotide duplications in flanking positions) has been interpreted to mean that these G + C clusters are mobile by an as yet unknown mechanism. Transposition of such elements would also be consistent with the observed polymorphism of mt genomes with respect to these G + C clusters [38]. Similar data from other fungi indicate that the phenomenon is wide-spread. A second element in *S. cerevisiae* mtDNA intergenic regions has been termed *ori* or *rep*, and there are still conflicting interpretations about its relationship to the mobile elements as opposed to an origin from mitochondrial promoters.

The expansion of A + T rich intergenic sequences can be explained by several plausible mechanisms. Slippage during replication or DNA repair could create tandem repeats, and subsequently unequal crossing over could lead to further expansion or contraction. From experiments in the laboratory of Clark-Walker [reviewed in Reference 28] it has been shown that relatively large deletions (3–5 kb) in intergenic regions have no consequences for mitochondrial gene expression and the ability of such strains to grow on nonfermentable substrates, and such regions may indeed be dispensable “junk” DNA.

An observation which was puzzling for a long time was that strains of *S. cerevisiae* produce spontaneous petite mutants at the high rate of approximately 1% per generation. The phenotype “petite” is caused by respiration deficiency which was shown to be due to deletions in the yeast mtDNA. It appears that these internal deletions are created by excisions occurring at directly repeated G + C clusters. This inference was made primarily from the comparison of the spontaneous generation of petites in

*S. cerevisiae* and in *Candida glabrata*. The latter has no such G + C clusters to destabilize the mt genome, and as a result the spontaneous rate of petite formation in *C. glabrata* is four orders of magnitude lower [28].

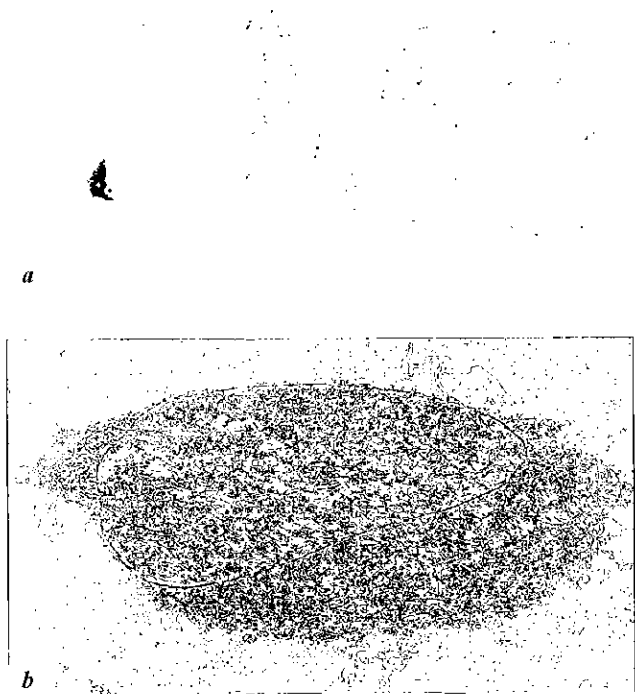
Linkages between genes, for example, *COXI-atp8-atp6*, have been preserved in many species, but evidence for rearrangements is abundant, and, most surprisingly, translocations have been found between interbreeding strains, calling into question the use of gene order in taxonomic classifications. Again direct repeats and recombination involving these repeats at distal sites may be responsible for the overall mechanism, and evidence for this "subgenomic pathway" has been accumulated by Clark-Walker and his coworkers [28]. In these experiments rearranged but complete genomes were deliberately produced, and no obvious deleterious effects on growth rates were observed. Such results could be explained if individual genes were expressed from individual promoters which are scrambled with the genes. As will be discussed later, there are multiple promoters on yeast mtDNA, and there are no recognized transcription termination sites, allowing constant levels of transcripts from rearranged genomes.

#### 4.1.5 Mitochondrial Genome in Kinetoplastid Protozoa

In organisms of the protozoan order Kinetoplastida a particularly unusual structure of mtDNA is found [39]. These "mitochondria" were detected by cytologists of the last century, but their unique morphology, cellular location, and assumed function caused them to be given a distinct name, kinetoplasts. The discovery of DNA in these organelles preceded the recognition that all mitochondria contained DNA because kDNA constitutes about 7% of the total cellular DNA, and hence is easily detectable by specific staining. Subsequently kinetoplasts were recognized to be highly specialized regions within the mitochondria, which were colocalized with the basal body of the flagella of these highly motile unicellular organisms (Figure 4.4A). Representatives of this group of organisms are the well known human pathogens *Trypanosoma cruzi*, *Trypanosoma brucei*, and a related organism, *Leishmania tarentolae*, isolated from the gecko (Figure 4.4).

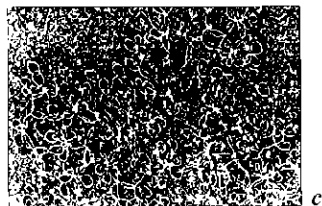
The kDNA exists of a network of ~40–50 maxicircles and 5,000–10,000 minicircles which are topologically linked by catenation. There appears to be some hierarchical order in the catenation of clusters of minicircles, the catenation of these clusters, and the catenation of the maxicircles into the entire network. Basket-shaped structures have been recognized, and the packing within the mitochondrion is by stacking and alignment of DNA fibrils, most likely with the help of specific proteins bound at regular sites. A combination of the activities of topoisomerases and restriction enzymes is employed to maintain order and to segregate these structures after DNA replication. Numerous reviews on the topology, packaging, and fractionation of these DNA circles are available [40,41].

The size range of the maxicircles extends from less than 20 kb in the African trypanosomes to about 35 kb in *Leishmania*, while the minicircles range from 645 pb in *Trypanosoma lewisi*, to 2500 bp in *Crithidia fasciculata*. Analysis of renaturation ki-



netics ( $C_0T$  analysis) with *T. brucei* DNA has indicated that kDNA has a total complexity of ~350 kb (the highest complexity observed among kinetoplastids), and this result immediately suggested that these circles must be quite heterogeneous. However, the gene content and even the gene order in the maxicircles in several species was shown to be identical. Size variation among species can be largely accounted for by the change in repeat number of a simple repeat in one variable region [41]. Thus, heterogeneity must be sought among the minicircles, and indeed, there are an estimated 400 different minicircles in *T. brucei* kDNA. The analysis is complicated by the fact that they are not found in equal copy numbers, and for two examples the copy





**Figure 4.4** A: Electron micrograph showing a thin section through *Leishmania tarentolae*. The basal body and axoneme extend to the left. B: Kinetoplast DNA. The maxicircle and many minicircles appear associated in an aggregate. C: Higher magnification view of many minicircles in various topological linkages. (Photographs provided by L. Simpson, UCLA.)

numbers were found to be about 60 and 500, respectively. In *Leishmania tarentolae* the complexity is lower and 17 minicircles make up all of the total amount of minicircle DNA in the UC strain, whereas approximately 80 minicircle sequence classes are found in the LEM125 strain. Recombination may contribute to the generation of minicircles, and at this time it seems impossible to give precise descriptions of their distribution in *T. brucei*. Another complication in the interpretation of renaturation kinetics is that all the minicircles have one conserved sequence of ~120 bp, and three conserved pairs of 18 bp inverted repeats.

The network of catenated maxi- and minicircles is highly sensitive to perturbations by intercalating molecules such as ethidium bromide or acridines. Treatment with such drugs causes loss of kDNA, and ultimately the loss of the capability to carry out mitochondrial respiration. Since respiration is required at least during part of the life cycle of these parasites, for example, in the insect host for the African trypanosome, the usefulness of such drugs in treatment and prevention of these tropical diseases has been explored extensively. It is also obvious that the replication of this network of DNA is intimidating to contemplate.

By now the complete sequences of the maxicircles of several species have been determined, and the genes present have been identified by homology with genes on mtDNA, some nuclear genes, and even chloroplast DNA. The challenge was not trivial, because there is extensive RNA editing in these species. The discovery of this process, not as a relatively rare oddity, but as a widespread phenomenon in the kinetoplasts, was one of the great surprises in molecular genetics in the 1980s, and the elucidation of its mechanism represents a major achievement (see Section 4.4. for further discussion) [42].

What genes were found? Not unexpectedly, kDNA maxicircles encode five subunits of NADH-CoQ reductase (complex I), cyt b of CoQ-cytochrome c reductase (complex III), three subunits of cytochrome oxidase (complex IV), one presumed subunit of ATPsynthase (complex V), and seven or eight unidentified reading frames.

Two ribosomal RNA genes yield rRNAs of 1150 and 610 nt (12S and 9S, respectively), which are exceptionally small, but still capable of forming secondary structures resembling the characteristic structures found in *E. coli* rRNAs. One would also expect to find tRNA genes, but RNA editing may obscure their identification by routine genome sequencing.

The maxicircles of some species have clearly been shown to encode some guide RNA (gRNA) genes, but many more gRNA genes can be found on the minicircles (see below).

Gene order is preserved between species where the analysis is complete, but sequence homology between species is recognizable (by computer and by cross-hybridization) only for genes whose transcripts are not heavily edited, while genes encoding heavily edited RNAs show little or no homology. A fertile field exists here for molecular taxonomists and evolutionary biologists, but the discussion exceeds the scope of this treatise.

The variable regions on the maxicircle between the ND5 gene and the 12S rRNA gene differ in size and sequence between species and even between stocks of *T. brucei*. It is unlikely to encode a functional protein, and speculation about its function centers around a role as replication origin.

The information or gene content of minicircles can be summarized only in very general terms, since their size, organization and complexity are all variables among species. However, all minicircles have a conserved sequence (at about the 90% level) of ~120 nt. Within this sequence a 13 nt sequence is recognized as a small gap in replicating minicircles, possibly in association with a small RNA. Hence its function in DNA replication is plausible. The presence of several stop codons and the lack of a start codon argue against any coding function within this region. A second common feature of minicircles is a number of runs of adenines at regular intervals, which give rise to a “bend” or “kink” in the DNA and cause such a molecule to have an abnormal electrophoretic mobility compared to a normal DNA molecule of identical size. The position can only be related to the position of the conserved 120 nt sequence, and where it has been examined, it appears to be at no particular distance from this landmark in different species. The binding of a protein at this location has been hypothesized to be related to the stacking of the circles within the kinetoplast.

The major class of transcripts from the minicircles are the small (<100 nt) guide RNAs. In some species (e.g. African trypanosomes) the gRNAs are from within a ~100 bp region flanked by 18 nt inverted repeats, a so-called coding cassette, but such cassettes are not recognizable in *T. cruzi* or *Leishmania tarentolae*. The role played by these gRNA in RNA editing will be discussed in Section 4.4.6.

#### 4.1.6 Mitochondrial Plasmids

In a previous section reference has been made to mobile introns in the yeast mt genome. Additional genetic elements found in the mitochondria of numerous fungi, protozoa and plants are small circular or linear plasmids. They have origins of DNA replication, replicate independently of the much larger mtDNA, and may even encode

some of their own replication machinery such as DNA polymerase. Some of these may become integrated into the mt genome, disrupting genes and making their presence felt by causing a recognizable phenotype. A listing of over 50 such plasmids in various fungi and higher plants can be found in a recent review on sexuality in mitochondria by Kawano and colleagues [43]. In some organisms such as *Neurospora* they may be the rule rather than the exception [44].

A number of these plasmids have been sequenced, but no significant insights about their biological function have become apparent. They range in size from ~1.5 to ~10 kb, with a few exceptions, the largest being the linear mF plasmid of *Physarum polycephalum* (~16 kb). They may be true molecular parasites adapted for autonomous replication and transmission with the mitochondrial genome. Thus, ORFs encoding DNA polymerase are commonly found, and RNA polymerases are also encoded by many plasmid genomes. When biological effects are observed, they may be incidental in most cases, since loss of the plasmid does not affect the organism's viability. The mF plasmid in *P. polycephalum* has been shown to encode a function required for developmentally regulated mitochondrial fusions during the life cycle of the slime mold, but strains missing the mF plasmid appear to be equally capable of sporulating or of forming a plasmodium.

The linear plasmids share several properties related to the mechanism of their replication, most notably the presence of terminal inverted repeats (TIRs) with proteins bound to their 5' ends. A replication mechanism resembling that of linear DNA viruses has been proposed, and further speculation centers around their origin as bacteriophages which have degenerated in the course of evolution. Since they are stable in the absence of selective pressure and have a high copy number, their use as vectors in yeasts has been proposed [45]. Transformation protocols using microprojectiles have been developed yielding transformants with useful efficiencies [46].

The replication of the circular plasmids has also been mostly speculated on, using information from bacterial systems as a guide. Replication intermediates described as  $\theta$  and  $\sigma$  [47] have been looked for in a few plants, and a rolling circle type of mechanism was found for the mp1 plasmid in the higher plant *Chenopodium album* (L.) [48]. In its most simplified formulation, the rolling circle mechanism of replication of circular genomes requires the following steps, first elucidated for bacteriophages such as  $\phi$ X RFI [47]:

1. Site-specific nicking within the origin creates a free 3'-OH group
2. Displacement of the 5' end from the circular template by a helicase, and complex formation with single-strand binding proteins
3. Extension of the 3'-OH end by DNA polymerase displaces more 5' single strand, and at this stage the molecule has the appearance of a " $\sigma$ " when viewed by electron microscopy
4. When continuous synthesis around the circular template has reached the origin, another cleavage of the displaced single strand creates a linear molecule which has to be circularized by ligation
5. Discontinuous synthesis converts the single-stranded circle into another double-stranded circle.

The cycle can repeat itself. By contrast, two replication forks moving in opposite directions on a circular DNA will create an intermediate with the appearance of a "θ" in the electron microscope.

Three significant biological effects have been associated with the presence of plasmids in mitochondria of fungi or plants.

#### 4.1.6.1 Fungal Senescence

Among the natural Hawaiian isolates of *Neurospora intermedia* the phenomenon of senescence was discovered, in contrast to the immortal nature of most laboratory and field-derived strains of *Neurospora*. A mitochondrial linear plasmid named kalilo (~9 kb) was found to be responsible. In juvenile strains kalilo DNA is harmless even at high copy number, but insertion into mtDNA appears to initiate the senescence process, and eventually almost all mtDNAs contain inserts at death. A systematic search uncovered a *Neurospora crassa* strain in India carrying a linear plasmid (maranhar) which also causes senescence by inserting into mtDNA, but kalilo DNA and maranhar DNA do not share any sequence homology. The investigation of 171 natural isolates of *N. crassa* and *N. intermedia* yielded another 28 senescing strains. Most strains, senescent or non-senescent, carried plasmids; the plasmids were linear or circular; some strains had more than one plasmid, even mixtures of linear and circular plasmids. A cross-homology study established sequence relatedness between plasmids from geographically distant origins, but a generalizable relationship of plasmids or sequences to senescence has not yet emerged. Thus, the current idea is that the plasmids act as insertional mutagens causing an abnormal mitochondrial physiology. However, the defect is not the result of an accumulation of random insertions at different sites; instead, a single mtDNA with a specific insertion predominates in a senescent culture.

The mechanism for the insertion may depend on the plasmid. Frequently the plasmid suffers short deletions, or base changes when inserted, and host sequences (mtDNA) may be duplicated in the form of long inverted repeats [44].

It should be pointed out that fungal senescence can also occur in the absence of any recognizable exogenous plasmid DNA as an inherent property of mitochondrial DNA itself [49]. This phenomenon is discussed in Chapter 7.5 (reviewed in References 44 and 49).

#### 4.1.6.2 Phytopathogenicity

As briefly summarized by Meinhardt and colleagues [45], the phytopathogenicity of fungi has been examined with the hope of correlating virulence and host range with the presence of specific plasmids. Some promising results have appeared, but far-ranging conclusions are not yet possible.

#### 4.1.6.3 Cytoplasmic Male Sterility

Different wild and cultivars of maize (*Zea mays*) mitochondria harbor a variety of plasmids: a linear 2.3 kb plasmid is found in all maize plants, while linear S-plasmids (S<sub>1</sub>, S<sub>2</sub>), and R-plasmids (R<sub>1</sub>, R<sub>2</sub>) distinguish different maize lines. Cytoplasmic male sterility (CMS) appears to be the consequence of the integration of the S-plasmids

into the mitochondrial genome, linearizing it in the process. The presence of the free plasmids (S or R) is not associated with CMS.

CMS is a common trait of higher plants characterized by abnormal pollen development. Maternal inheritance suggests in general a mitochondrial mutation. Such mutations may be created by plasmid integration, or they may result from aberrant recombination events involving the multiple circular mt genomes present in plant mitochondria [50]. The situation is complicated by the possibility of suppressing male sterility and restoring normal pollen development when specific nuclear genes (*Rf*: restorers of fertility) are present. Molecular mechanisms for these phenomena are still elusive. The analysis is made especially challenging by the large size of plant mt genomes. CMS as a result of chimeric genes created by rare recombination events is discussed further in Chapter 8.

## 4.2 NUCLEAR GENES ENCODING MITOCHONDRIAL PROTEINS

The number of different polypeptides in each mitochondrion has been estimated to be of the order of one thousand [51]. This also means that of the order of one thousand nuclear genes contribute to the biogenesis and function of mitochondria. Many of these are known, but many remain to be identified and characterized. Logically one can subdivide this large group of genes and their proteins into two major groups: 1) genes necessary for the biogenesis of normal, fully functional mitochondria, and 2) genes encoding proteins, which play a role in the various specialized biochemical activities of mitochondria. An exhaustive list will not be presented here, but the major categories and a few examples will exemplify the two groupings and provide a logical starting point for the discussion of several aspects of the biogenesis of mitochondria that have not yet been presented. A discussion of the biochemical reactions localized in these organelles is presented in Chapter 6.

### 4.2.1 Enzymes Required for Mitochondrial Gene Expression

In past chapters many references have already been made to enzymes required for the replication of the mitochondrial genome, its transcription, the processing of the primary transcripts, and the translation of mature mRNAs. At least one DNA polymerase has been identified, belonging to the polymerase  $\gamma$  family [52]. Primers for replication are made by an RNA polymerase, and one can anticipate the need for a ligase to make covalently closed, supercoiled circles. Mitochondrial topoisomerases have not been described. Enzymes involved in DNA repair and recombination are less well characterized, and the relative importance of these activities is variable in different organisms. In this context one might also mention that there are no histones in mitochondria, although mitochondrial DNA appears to be complexed to a protein which resembles the bacterial HU protein or the eukaryotic nuclear HMG1 proteins [53,54]. For transcription, an RNA polymerase and at least one transcription factor or activator are required.

The number of activities associated with RNA processing, splicing and editing depends on the species, although several types of nucleases for the cleavage of poly-

cistronic RNA and the maturation of tRNAs are likely to be found in all mitochondria. Polyadenylation of RNA transcripts in metazoan mitochondria requires at least one enzyme.

The translation of mitochondrial mRNAs will be discussed more extensively in Section 4.5. Ribosomal RNAs are universally encoded by mtDNAs, as are some, but not all the tRNAs required. Similarly, in metazoan mitochondria all the ribosomal proteins are imported (and hence nuclear-encoded), while some organisms (notably plants and fungi) have some of their r-proteins still encoded by mtDNA. Specific as well as nonspecific translation initiation factors and presumably non-specific elongation factors will be discussed in Section 4.5.5; all are encoded by nuclear genes. The aminoacyl-tRNA activating enzymes are also all imported.

The complicated nature of mitochondrial gene expression may be illustrated with the finding that 18 nuclear genes have been shown to be required for the expression of the yeast mitochondrial gene encoding cytochrome c oxidase subunit 1 [55].

In general, one would expect a mutation in any of these nuclear genes, especially a deletion or null mutation, to have serious consequences for the biological function of mitochondria. Such mitochondria would be incapable of respiration, but may continue to perform other important functions such as heme synthesis, lipid synthesis, amino acid synthesis, and nucleotide synthesis. DNA replication is not absolutely required for the biogenesis, since mtDNA-less ( $\rho^0$ ) mitochondria have been described for yeast and mammalian cells. Similarly, mitochondrial protein synthesis can be completely abolished by mutations, without affecting the continued assembly of this organelle in mammalian cells [56,57]. Therefore, replication, transcription and translation seem to serve exclusively the purpose of maintaining and assembling a functional electron transport chain and oxidative phosphorylation mechanism, a vital but by no means the only function of mitochondria.

The contribution of large number of genes (and proteins) is required for the import of the vast majority of mitochondrial proteins into the matrix, into the two membranes, and into the intermembrane space. This subject is dealt with in Section 4.6, where the function of the various proteins is explored in detail. In broad categories one can list the proteins of the import machinery in the outer and inner membranes (receptors, formation of import pore or channel), proteases/peptidases responsible for protein maturation, and mitochondrial heat-shocklike proteins (chaperones) which assist in the conformational transitions of the imported peptides. Cytosolic chaperones are equally essential, although they do not belong exclusively to mitochondria. It is easily understood that a serious defect in any of these major factors required for protein import will eliminate not only respiration and oxidative phosphorylation, but will affect mitochondrial biogenesis in general and may therefore be lethal.

Many if not most of these proteins have been discovered by a genetic approach, and for obvious reasons the majority of the corresponding genes have been identified in the yeast *Saccharomyces cerevisiae*. This yeast is a facultative anaerobe, and hence is capable of growth without respiration in the presence of abundant glucose for glycolysis. A variety of clever and elegant screens have been developed for the identification of yeast mutants with defects in mitochondrial biogenesis. Historically the first mutants were the so-called nuclear *petite* or *pet* mutants. *Petite* (small) colonies arise when a respiration-deficient yeast mutant is grown on glucose: normal colonies will

continue to grow even after the glucose is exhausted, because they are capable of metabolizing the ethanol accumulated during the first phase of growth. The first *petites* discovered had “cytoplasmic” mutations, later identified as large deletions or complete loss of mtDNA. Nuclear petites have the same phenotype due to a nuclear mutation affecting respiration. They can be most readily distinguished with the help of a tester strain lacking mtDNA ( $\rho^0$ ). After fusion and formation of diploid cells, the diploid cells will grow in the absence of a fermentable carbon source if the mutation is nuclear and recessive.

The selection of mutants after mutagenesis makes use of replica-plating techniques. It can also be combined with selections at permissive and nonpermissive temperatures to obtain conditional mutants. Additional wrinkles in the design of selections have been described, but the reader is referred to the original literature for details. Once a *pet* mutant has been identified, a number of methods are available to establish some basic facts: is it a new mutation, and what kind of mutation is it? Complementation analysis with existing, well-characterized mutant panels is one option, but it may be quite laborious, since several hundred complementation groups have already been identified. Cloning of the complementing gene from a yeast DNA library is probably a more efficient means of obtaining a sequence which can be checked against the existing yeast genome data base [58]. An expert and comprehensive review of the *PET* genes of *Saccharomyces cerevisiae* has been written by Tzagoloff and Dieckmann [59]. Not surprisingly, the collection of *pet* mutants includes many mutations in nuclear genes encoding subunits of the mitochondrial electron transport chain. The activity of specific complexes is affected. Less expected was the finding of mutants with defects in a specific complex, but the defective gene was not a structural gene for a particular subunit. Instead, further analysis has revealed that the assembly of the various complexes requires additional proteins. Only a few examples will be mentioned here, with additional details to be presented in the discussion of the assembly, structure and function of each complex in Chapter 5. *COX14* is a gene whose product, Cox14p, is required for the assembly of cytochrome oxidase (complex IV) [60]. A newly discovered member of a family of ATPases is required for the assembly of several complexes including complex V [61].

Many *pet* mutants have a pleiotropic phenotype, and are often found to be defective in mitochondrial protein synthesis. Others are defective in heme synthesis, lipoic acid synthesis, coenzyme Q synthesis, or in enzymes of the Krebs cycle.

The first mutants specifically isolated to be defective in mitochondrial protein import were described by Yaffe and Schatz [62], and many more have been identified in the intervening years [63–65]. A detailed discussion follows in Section 4.6.

Finally, proteins and their nuclear genes required for the many biochemical reactions in mitochondria are too numerous to mention. Every student of Biochemistry is introduced to the enzymes of the tricarboxylic acid cycle (Krebs cycle), and a defect in one of these will also lead to a respiration-deficient phenotype. The Krebs cycle reactions are also responsible for the interconversion of various four carbon and five carbon molecules which by trans-amination yield amino acids such as aspartate and glutamate. The urea cycle, fatty acid metabolism, and a number of other biochemical pathways require many enzymes which have to be imported into mitochon-

dria. Defects in any of the latter enzymes may have serious consequences at the organismic level, but may not be recognizable as mitochondrial defects in the sense of perturbing the biosynthesis and morphology of this organelle.

There will undoubtedly be many more genes and gene products not mentioned in the brief survey so far. They may be associated with processes already mentioned, or may be relevant for the generally less familiar pathways in mitochondria, for example, the biosynthesis of cardiolipin. A number of the more interesting ones will be discussed under specific headings such as the mitochondrial permeability transition and apoptosis. One could also speculate about proteins involved in fusion and fission, in the organization of the cristae and matrix, and in the association of mitochondria with the cytoskeleton.

## **4.2.2 Nucleomitochondrial Interactions**

### **4.2.2.1 Introduction**

The existence of two spatially separated genomes, each contributing, however asymmetrically, to the biogenesis of mitochondria, has raised for a long time already questions about “interactions” between the two genomes. The focus of these questions can vary. One may wonder how the number of mitochondria in a cell is maintained over many cell divisions, or how it is possibly reduced or expanded, as appears to be the case in oogenesis. Is mitochondrial DNA replication in any way coupled to nuclear DNA replication to assure a more or less constant ratio between the two genomes? How is the composition in different cell types altered? Most obviously, there are differences in the morphology of the cristae or in the relative surface area of the inner mitochondrial membrane, which may reflect differences in respiratory activity in different tissues. It may be conceptually easy to understand that one can express the enzymes for the urea cycle at different levels in different tissues and hence have differing specific activities in mitochondria. On the other hand, to downregulate or upregulate the activity of, for example, cytochrome oxidase, requires not only a coordinate regulation of at least 13 genes in mammals, but three of these are on the mt genome, while the other ten are in the nucleus. If one wishes to regulate oxidative phosphorylation and electron transport, should all complexes be up- or downregulated, that is, are the nuclear genes encoding subunits for complexes I–V coordinately expressed? Or is there a rate limiting complex (a “bottleneck” in electron transport), whose level determines the rate for the overall pathway? The mitochondrial genome is transcribed into polycistronic transcripts, but the translation of individual processed mRNAs may still be controlled separately to provide subunits at different levels. It is also conceivable that there is no stringent coordination of the synthesis of different subunits of a complex, but one critical subunit is made in controlled, limiting amounts, assembly takes place and all other excess subunits are turned over rapidly.

It has already been mentioned that a crucial factor in the replication and expression of the mtDNA in mammals is the mitochondrial transcription factor, mtTFA [66], encoded by the nuclear genome (see Section 4.4.1). Regulating the availability of this factor might be sufficient to control the expression of mitochondrial genes, and if the



expression of mtTFA were coordinately regulated with the expression of other nuclear "mitochondrial" genes, a simple mechanism suggests itself.

A number of different approaches to answer questions related to nucleomitochondrial interactions have been taken. Again, yeast has led the way with the exploitation of powerful genetic techniques. In mammals and plants the number of relevant mutants is quite limited, even with cell cultures, and alternate means must be sought. The two systems will therefore be discussed separately.

#### **4.2.2.2 In Yeast: *Saccharomyces cerevisiae***

Yeast is not only a good model system because of the advanced state of genetics of this system, but also because yeast cells can be experimentally manipulated in a way that directly addresses the regulation of the biogenesis of mitochondria. When grown in a medium in which glucose is abundant (YPD (dextrose) medium), the synthesis of the electron transport chain is almost completely suppressed, and the cells use fermentation (glucose  $\rightarrow$  ethanol) exclusively to satisfy their energy needs. The morphology of the residual mitochondria (with normal levels of mtDNA) is quite abnormal, making the mitochondria barely recognizable by the nonexpert. Almost immediately after a switch to a nonfermentable carbon source such as glycerol, ethanol or acetate, there is a rapid induction of the transcription of many genes, including those encoding subunits of the electron transport chain complexes. The phenomenon is referred to as "glucose repression." In general it also includes the repression of non-mitochondrial enzymes involved in the uptake and utilization of carbon sources, of which invertase (*SUC2*)<sup>1</sup> is a prominent example, since this gene has been used extensively in the genetic analysis of glucose repression, and results obtained with this gene have been extrapolated to other genes. A number of expert, recent reviews have been written on this subject [67–70]. A possible physiological explanation is that it is more economical for the yeast cell to "waste" ethanol and not invest extensively into the biogenesis of the inner mitochondrial membrane. It may also be that growth in the absence of respiration is preferred under these conditions because it saves the cells from the potential onslaught of reactive oxygen species. However, in the course of evolution yeast has learned to adapt rapidly to changing conditions, and when transferred from rotting fruit to a much more dilute aqueous environment, a switch to the more efficient use of a limiting carbon source is expected to be advantageous. Another example of changing environmental conditions encountered by such microorganisms is a change in oxygen availability. Thus, oxygen deprivation also results in changes in gene expression including those encoding mitochondrial complexes [reviewed in Reference 71].

Until recently all responses of yeast to glucose levels or oxygen pressure were interpreted in terms of transcriptional regulation. A signaling pathway for glucose repression can be decomposed into the following major subdivisions:

<sup>1</sup>Following the convention in yeast molecular genetics, genes will be designated by italicized, capital letters (e.g., *SDH2*), while their respective peptides or protein will carry the same name with only the first letter capitalized, followed by a p (e.g., Sdh2p).

1. Glucose must be taken up by the cell and it itself or a metabolite must be sensed by a key protein in the pathway.
2. The signal must be transmitted through the cytoplasm via a series of protein-protein interactions (which may involve protein kinases and phosphatases), or by second messengers such as cAMP,  $\text{Ca}^{2+}$ , or inositol phosphate derivatives.
3. Final targets of the signal transduction pathway are nuclear transcriptional activators or repressors that regulate the transcription of specific genes.

Among the major goals of research in this area therefore have been the characterization of the postulated specific transcription factors and the identification of the corresponding cis-acting sequences (upstream activating sequence or UAS, and upstream repressor sequence or URS) of the genes in question. There may be more than one UAS, and they may be distinguished as UAS1A and UAS1B, for example. A more explicit description of the transcriptional activators or repressors and their interaction with the basic transcriptional machinery (RNA polymerase, TATA-binding protein, TFIID, and initiation factor TFIIB) will not be attempted here. Broadly speaking, an activator/repressor protein has a DNA binding domain for specific interactions with target DNA sequences, and another domain for interaction with the transcriptional initiation complex. In some cases these domains are on the same peptide, while in others different subunits of a complex contribute these distinct domains. There may also be another domain serving a regulatory function (see below). Pioneering research on the regulation of the cytochrome c gene in yeast (*CYC1*) was carried out by the group of Guarente [72,73], and the cumulative conclusions of the Guarente laboratory can illustrate some general principles. First, the *CYC1* promoter region was found to contain several CCAAT boxes which were identified to be the target of the Hap2/3/4/5 complex. This heterotetrameric complex is a transcriptional activator whose activity is low in glucose-rich medium and high in glycerol-containing medium. It is one of the targets in the signal transduction pathway initiated by the uptake and phosphorylation of glucose. Glucose not only controls its activity (via phosphorylation or dephosphorylation), but it also controls the transcription of some of the *HAP* genes themselves. Not all CCAAT boxes are equal, and in the *CYC1* promoter one of the distal, upstream CCAAT boxes is the target of the Hap1 transcription factor whose activity is controlled by oxygen, via heme as an intracellular oxygen sensor [71,73–75].

It is not yet clear whether all genes encoding subunits of respiratory complexes are regulated by Hap1p and or the Hap2/3/4/5 complex, but the required CCAAT boxes have been identified in the *SDH2* gene of complex II [76], and the *SDH3* gene was cloned specifically on the expectation that it was transcriptionally regulated by the Hap2/3/4/5 complex and glucose [77]. Cyb2p is a lactate cytochrome c oxidoreductase localized in the intermembrane space. Expression of *CYB2* is repressed by glucose and dependent on heme. Its promoter has been characterized by Lodi and Guiard [78] and shown to have two positively acting cis elements and one negatively acting cis element. One of these binds the Hap1 (Cyp1) activator, and the Hap2/3/4/5 complex is another regulatory factor for this gene. Not all the genes studied to date

are regulated by both Hap1p and the Hap2/3/4/5 complex. Among those subject to both are *CYC1*, *CYB2*, *CYT1*, *QCR2*, and *COX6*, all genes encoding peptides in the electron transport chain complexes and therefore useful in the presence of oxygen and required with a nonfermentable substrate. Several genes for enzymes/peptides of the Krebs cycle are regulated by the Hap2/3/4/5 complex alone (the target of glucose signaling), while another subset of enzymes needed for heme, sterol or fatty acid biosynthesis are regulated by Hap1p alone [see Reference 75 for specific references].

Hap1p, in addition to DNA binding and transactivation domains also has a domain for heme or metal binding. It is speculated that the redox state of the cell, depending on the level of oxygen, is sensed via the heme, and heme binding unmasks the DNA binding domain. The activator is therefore active only in the presence of oxygen. The activity of Hap1p may be further modulated by the specific DNA sequence to which it binds, that is, its activity is found to be promoter-dependent. One hypothesis to explain the behavior of Hap1p postulates allosteric interactions between the various domains. Since Hap1p also controls heme biosynthesis, the possibility exists for the establishment of complex feedback loops and very intricate fine tuning of gene expression to optimize activity to a given environment.

The Hap2, Hap3, and Hap4 peptides form a heterotrimer in which Hap2 and Hap3 domains control assembly and interaction with the 5'-ACCAATNA-3' sequence, and Hap4p contains the trans-activation domain. The assembly and/or activity may be controlled by a kinase (Snf1p), but the transcription of the *HAP2* and *HAP4* genes is repressed by glucose. Thus both the level and the activity of the complex are under control by the carbon source, and perhaps even by oxygen, since it has been suggested that heme may influence the translation of the *HAP4* mRNA.

Another gene with a prominent role in the control of the expression of respiratory chain peptides and related functions is the *ROX1* gene. The transcription of this gene is dependent on heme, and the Rox1 protein acts as a transcriptional repressor of a variety of genes for mitochondrial proteins. A challenging problem in yeast has been the finding of isoforms of proteins encoded by gene pairs. Some examples are *COX5A* and *COX5B*, *CYC1* and *CYC7* [75]. Their expression is alternate, that is, when one is expressed under aerobic conditions, the other is expressed under anaerobic or hypoxic conditions. The preferential expression one or the other cytochrome c or cytochrome oxidase subunit may again reflect an optimization and adaptation to a variety of conditions, to transient conditions in shifting from respiration to fermentation, and to different levels of availability of heme. *HAP1* and *ROX1* may therefore play antagonistic roles in the precise control of the ratios of these isoforms.

Although the number of genes for mitochondrial proteins which have been explicitly examined is limited, one could probably draw the conclusion with some confidence that many if not most of these will have promoter elements recognized by either the Hap1 peptide or the Hap2/3/4/5 complex, and therefore a basis for the coordinate expression of many genes under conditions of glucose deprivation is emerging. Additional factors and their DNA targets, such as Rox1p, may be discovered, and they may be required for fine tuning of gene expression and precise adjustments to the available carbon source, oxygen pressure, and other conditions. By regulating

Hap1p and Hap2/3/4/5p activities mitochondrial biogenesis can be controlled as a whole. When the availability of glucose is the only external factor, it remains to describe the cascade of signals initiated by this metabolite and interpreted eventually by nuclear factors. A large volume of publications have addressed this problem, and only a bare outline of the upstream signaling pathway can be presented in the present context.

There is agreement that glucose has to be phosphorylated, but uncertainty begins already in the distinction between a signal from glucose 6-phosphate or one of its metabolites, or a signal via an allosteric mechanism involving the hexokinase. Glucose addition to yeast has also been shown to activate the RAS-adenylate cyclase pathway [e.g., 79], but current thinking does not include this pathway in the phenomenon of glucose repression. Genetic analysis has quite definitively implicated the product of the *REG1* gene in the pathway, and Reg1p has recently been identified as one of possibly several regulatory proteins for the major protein phosphatase in yeast encoded by the *GLC7* gene [80]. A protein kinase definitely implicated in the pathway is the Snf1 kinase [69,81]. It is not yet clear, however, whether the Snf1 kinase phosphorylates transcriptional activators such as Hap2/3/4/5p directly, or whether there are intervening steps. Similarly, the target in this pathway of the Glc7 phosphatase has not been determined. Recent reviews on glucose repression should be consulted on the latest developments in this active field [67–69,82].

Until recently, most attention was devoted to understanding how gene expression is regulated by the carbon source at the transcriptional level. A new dimension to the analysis of this phenomenon was added by studies on the steady state levels of the mRNAs for the iron-sulfur subunit (Ip) and the flavoprotein subunit (Fp) of complex II in yeast. Transcriptional regulation plays a role, but an equally important mechanism was found to be the control of the half life of these transcripts by the carbon source [76,82,83]. In medium with a nonfermentable carbon source the mRNAs have long half-lives, contributing to the high steady state levels of these mRNAs and therefore to the rapid synthesis of the SDH peptides. In the presence of abundant glucose the half-life of these mRNAs is very short (<5 minutes), and the addition of glucose to an induced culture causes an extremely rapid turnover of these specific mRNAs. As a result, steady state levels are maintained at a very low level in glucose by a combination of repression of transcription and rapid turnover of the transcripts. From another point of view, a three to fourfold induction of transcription and a stabilization of the message in glycerol leads to a 12- to 20-fold induction at steady state.

The mechanism of this very specific destabilization of a subset of mRNAs in glucose has been partially elucidated [82,83]. The important cis-acting element in the Ip-mRNA is the 5' untranslated region. The addition of glucose triggers a very rapid decapping of this mRNA, followed by an equally rapid degradation by the 5'-3' exonuclease (Xrn1p). Since these activities take place in the cytosol, it is not surprising that various nuclear factors such as Hap2/3/4/5p are not required. More surprising was the finding that the Snf1 kinase was also not required for this glucose-triggered degradation mechanism. On the other hand, a phosphorylation/dephosphorylation step or cascade is indicated by the requirement for the Reg1 protein, a phosphatase regulator.

Theoretical considerations and observations with a temperature-sensitive eIF3 mutant have focused attention on a competition between different events at the 5' end of the mRNA [83]. On the one hand, initiation factors (eIFs), the 40S small ribosome, and F-met-tRNA will bind the 5' UTR at the cap and then translocate to the start codon where the 60S ribosomal subunit is added and peptide elongation begins. At the same time a decapping activity is present [84], aiming for the same 5' end of the transcript. Once decapped, the mRNA is not only no longer a good target for the initiation factors, but is also a good substrate for the constitutively active 5' exonuclease. A working hypothesis therefore proposes that glucose influences the outcome of this competition in favor of the degradative pathway. These observations fit into a more general picture of mRNA turnover developed over the past few years [85–87]. Constitutively short-lived mRNAs are susceptible to a rapid shortening of their poly(A) tails, which precedes the decapping. The rate controlling step appears to be deadenylation, which in turn is controlled by a specific nuclease [88]. The activity of this nuclease is regulated by its interaction with other factors binding to sequences within the mRNA, which are responsible for the specificity of this process. Recent experiments have emphasized that the 5' end and the 3' end (poly(A) tail) of mRNAs interact, most likely indirectly via proteins such as the poly(A) binding protein, eIFs, ribosomal proteins and other factors yet to be characterized [89–91]. This interaction may have a major influence on either the stability of an mRNA, or on the efficiency with which it is translated.

The signal generated by glucose could therefore have as its cytoplasmic target a factor influencing the deadenylation reaction. Alternatively, it may directly modify one of the initiation factors and hence affect initiation, and hence decapping, without an obligatory deadenylation. A precedent for mRNA turnover in the absence of deadenylation is the turnover of mRNAs with premature stop codons by the UPF-mediated pathway [91].

Post-transcriptional mechanisms as well as transcriptional regulation must be considered in yeast when the regulation of respiratory capacity and assembly of the oxidative phosphorylation system in yeast mitochondria is under discussion. One can speculate whether such a multitude of mechanisms is required for fine tuning, for establishing the overall range of the activities involved, or for a rapid response to rapidly changing environmental conditions. It will be important to keep these considerations in mind when similar questions are raised about various mammalian cell types in intact organisms.

#### ***4.2.2.3 Regulation of Nuclear Respiratory Genes in Mammalian Cells***

The general considerations and the observations made with yeast have encouraged hypotheses and experiments aimed at similar goals for mammalian cells: characterization of transcription factors and their targets on the relevant promoters, which might explain the coordinate expression of the large number of nuclear genes encoding mitochondrial proteins, and the nuclear-mitochondrial interactions which are assumed to take place to make nuclear gene expression match mitochondrial gene expression or vice versa. A genetic approach is technically and logistically difficult if not impossible with diploid mammalian cells, and no conditions are known in tissue culture

which would induce or repress the biogenesis of mitochondria to permit willful manipulation of the experimental system. A priori one would not expect mammalian cells in culture to be able to respond like yeast to changing carbon sources, since they have become adapted in evolution to a very stable environment in vivo and hence have no need regulate their mitochondria over a short term. Experiments with Chinese hamster fibroblasts have shown that a large fraction >50% of the energy needs of these cells is satisfied by glycolysis [92], and even when glucose is deliberately limited in culture, there is no induction of transcripts of complex II, for example [57], and only a modest increase in respiration. On the other hand, it has become apparent, that different tissues and cell types may have widely differing energy needs and hence may have to adjust their level of mitochondrial respiration and oxidative phosphorylation to these needs as part of the process of differentiation. From the study of mitochondrial diseases and the discovery of the differential sensitivity of different tissues to mutations in mtDNA, neurons and muscle cells were deduced to be the most active tissues, as already anticipated from their physiological functions. Cardiac muscle is a specific example of a tissue deriving most of its ATP from respiration, and, not surprisingly, cardiac myopathies are prominent among the clinical manifestations associated with mutations in mtDNA.

To repeat, one may not expect to find rapid, short-term fluctuations in mitochondrial biogenesis in a given cell type, but one can anticipate that the expression of a large number of genes encoding respiratory and other mitochondrial proteins may be expressed at a higher level in some cell types compared to others, and thus the existence of common regulatory molecules may be the simplest mechanism for a coordinate expression.

The number of mammalian genes investigated fully is still small. For practical reasons cDNA clones are the first to be isolated, but the step to the full genomic clone, or at least the promoter region is not always taken. From the few examples available one may tentatively conclude that there is no single universal cis-acting sequence element with its associated transcriptional activator which is common to all such genes. However, at least two factors have been characterized which play key roles in the regulation of entire subsets of genes. Which genes belong to the set, and why, is not yet clear.

Evans and Scarpulla [93,94] were the first to define a factor, designated as NRF-1 (nuclear respiratory factor 1), from their analysis of the promoters of the mammalian (rat) cytochrome c and cytochrome oxidase subunit Vb (COXVb). Sequence analysis of the cytochrome c promoter revealed the presence of several consensus sites for well-known transcription factors such as Sp1, CCAAT, and the cAMP response element binding protein CREB. In addition, a new cis-acting element was defined as a palindromic motif consisting of tandemly repeated GC sequences over a single helical turn which interacts with the NRF-1. The case was strengthened when over the years several other nuclear genes encoding mitochondrial proteins were found to have the same element (COXVb, COXVIIaL, COXVIc, mtTFA, an ubiquinone binding protein, a mtRNA processing enzyme, aminolevulinate synthase) [95], prompting Scarpulla and his colleagues to purify the human protein to near homogeneity from cultured cells. Partial peptide sequences were used to synthesize degenerate primers which in turn led

to the isolation of full length cDNA for NRF-1 and the production of authenticated recombinant protein in bacteria [96,97]. NRF-1 is a single peptide with a novel DNA binding domain (related to DNA binding domains in two developmental regulatory proteins found in *Drosophila* and the sea urchin), and a transactivating domain. The finding of an NRF-1 site in the promoter for the mitochondrial transcriptional activator (mtTFA) is of special interest. Since mtTFA is required for both replication and transcription of mtDNA (see Section 4.4.1), one can begin to build a case for a role of NRF-1 in the nuclear control of mtDNA copy number and gene expression. Aminolevulinate synthase is also not an arbitrary, indifferent enzyme for this discussion. It is the first and rate-limiting enzyme in the pathway of heme synthesis in the matrix. Although a role for heme in the control of expression of mammalian cytochromes is not yet as well defined as in yeast, the connection is tantalizing.

When several other promoters of respiratory genes, for example, COXIV, either failed to show an NRF-1 binding site or could not be footprinted with pure NRF-1, the search for additional recognition sites and factors by Virbasius and Scarpulla [98,99] succeeded in the identification of a second factor termed NRF-2. The core GGAA motif was somewhat familiar from the previous characterization of the ETS-domain family of transcription factors, but when the NRF-2 site from COXIV was used in an affinity column, a novel DNA binding activity could be purified [100]. The DNA binding activity copurified with five distinct polypeptides ( $\alpha$ ,  $\beta_1$ ,  $\beta_2$ ,  $\gamma_1$ ,  $\gamma_2$ ). The  $\alpha$  subunit was subsequently identified as the DNA binding peptide, but it is found as a heteromeric complex with one of the other peptides ( $\alpha\beta_1$ ,  $\alpha\beta_2$ ,  $\alpha\gamma_1$ ,  $\alpha\gamma_2$ ). These complexes differ in their affinity for the NRF-2 site, and to tandem NRF-2 sites, a characteristic that had already been observed for the related ETS-domain transcription factor GABP of the mouse. Further cloning and sequencing of all five human NRF-2 subunits established NRF-2 as the homologue of the mouse GABP, a widely expressed transcription factor first defined by the study of viral promoters. Consensus sequences for the NRFs are the following:

NRF-1: ...YGC**G**CAY**G**CGCR...; NRF-2: ...ACCGGAAGAG...

Reminiscent of the Hap2/3/4/5 complex in yeast, NRF-2 has DNA binding domains and transactivation domains on separate peptides. In addition, the affinity of some of the heterodimers for a promoter can be further enhanced by cooperative binding to tandem NRF-2 motifs, and overall a mechanism is created to modulate promoter strength for different genes.

It was also first found by Scarpulla and his coworkers [101] that some genes have both NRF-1 and NRF-2 motifs which contribute to the control of expression of these genes. Another recent example was found in the promoter for the iron-sulfur subunit of succinate dehydrogenase (and complex II) [102,103]. In this promoter the two sites are within 200 nucleotides upstream from the transcription start site, and mutagenesis of either site reduced the expression of a reporter gene from this promoter five- to tenfold. Both NRF-1 and NRF-2 were shown to footprint the respective sites independently. Other promoter elements were found further upstream (Sp1, CCAAT), but

deleting them had no significant effect on the expression of a reporter gene in fibroblasts, myoblasts, or differentiated myotubes.

The notion of having a limited, select number of transcription factors and promoter sequences exclusively devoted to the transcription of respiratory genes and mitochondrial proteins is apparently too simple. Several such genes have been found to lack NRF-1 or NRF-2 sites altogether (based on sequence inspection), while other genes unrelated to mitochondrial function contain such sites in their promoter region [95].

Mammalian cytochrome oxidase (COX, complex IV) consists of 13 subunits of which ten are encoded in the nucleus. It has been intriguing that three of the nuclear-encoded subunits (VIa, VIIa, and VIII) exist as multiple isoforms which are differentially expressed in different tissues. The L form is named after its identification in liver, and this form is also found in many other tissues. The H form is found predominantly in skeletal and cardiac muscle. A detailed analysis of the COXVIaH promoter region with a reporter construct in transgenic mice as well as in tissue culture has verified the highly specific elevation of expression in heart and muscle, with some activity in the brain, but virtually none in other tissues. Expression of this gene was suppressed in myoblasts, but dramatically induced when these myoblasts were induced to differentiate into myotubes. A 300-bp promoter segment was entirely sufficient to mimic the expression of this gene and its dependence on muscle differentiation. No NRF-1, NRF-2, or other element previously associated with respiratory genes (see above) was found in this region. Instead, an MEF2 consensus sequence and an E-box element were shown to be essential by mutational analysis [104]. The muscle enhancer factor (MEF) comprises a family of four genes induced by myogenic HLH proteins (myo-D, myogenin, Myf5, and MRF4), and MEF2 has been demonstrated to be significantly involved in muscle differentiation [see References 105 and 106 for summaries and examples]. Thus, the activation of the COXVIaH gene during muscle cell differentiation is understandable from a consideration of its promoter, but the promoter does not betray that the protein functions in mitochondria.

A very similar analysis was made with the SDH2 promoter (iron-sulfur subunit of complex II). Transcripts of this gene are also significantly elevated in adult skeletal and cardiac muscle. In contrast to COXVIaH, however, the expression of the SDH2 gene was not upregulated upon myotube formation, consistent with the absence of promoter elements responding to myogenic transcription factors. If there is no general, coordinate induction of respiratory chain peptides, one may wonder about the purpose of inducing COXVIaH mRNA by at least an order of magnitude in myotubes compared to myoblasts. It has been proposed from experiments with the homologous subunit in yeast that this subunit is responsible for sensing ATP and phosphate levels, that is, for the modulation of enzymatic activity of complex IV under varying metabolic conditions [107]. For this factor to participate in the regulation of cytochrome oxidase activity requires one of two possible mechanisms: either the subunit VIaH replaces the pre-existing subunit VIaL directly, or there is turnover of the entire complex, with newly made complex including the muscle-specific VIaH subunit.



Thus, at first sight, the well-controlled and already thoroughly investigated rat or mouse myoblast/myotube system in tissue culture may serve as a good model system in which to study mitochondrial biogenesis and the control of respiratory gene expression. Much previous attention has been focused on muscle specific proteins such as creatine kinase and the various cytoskeletal proteins of the sarcomere. On the other hand, the preliminary lessons are that more subtle mechanisms for modulating mitochondrial respiration may be at work, requiring only a limited number of changes in the composition of the electron transport complexes. A second, and very interesting, preliminary conclusion is that myotube differentiation in culture is incomplete, in the sense that maturation, stimulation, and contractile activity of a myofibril may be required for further changes in respiratory gene expression and mitochondrial capacity.

Two other characterizations of genes had once raised expectations about finding more universal promoter elements which might be unique or specific for mitochondrial proteins. The analysis of the promoters for the ATP/ADP translocator and for the  $\beta$  subunit of the F1-ATP synthase had identified so-called OXBOX and REBOX elements which were postulated to respond to nuclear factors present at variable levels in myoblasts, myotubes and HeLa cells [108,109]. A generalization of these conclusions awaits the characterization of more genes in this category, but at this time one can state that many other relevant genes already analyzed do not contain these particular elements as a recognizable consensus sequence.

#### ***4.2.2.4 Coevolution of Nuclear and Mitochondrial Genomes***

From the above description it is clear that several dozen nuclear-coded proteins interact either with the mitochondrial genome (replication, transcription, etc), or directly with proteins encoded by the mt genome. The mt genome suffers sequence substitutions at a significantly faster rate than the nuclear genome. If altered proteins result from mtDNA substitutions, compensatory changes may have to occur in the nuclear genome to assure proper strong, functional interactions between the gene products, in forming a complex of the electron transport chain, for example. Thus the two genomes are expected to coevolve, and strong evidence for this concept has been deduced from studies with interspecific hybrids and cybrids of mammalian cells. The first experiments addressing this issue were reported by Clayton and colleagues [110] from their analysis of mtDNAs in human-mouse interspecies hybrids. Further pioneering studies of Wallace and Eisenstadt [111] indicated quite convincingly that the nuclear and mitochondrial genome must be compatible, i.e. there is species specificity, and a human mtDNA cannot coexist for long in a rodent cell, or vice versa. More specifically, in the Scheffler laboratory, it was found that nuclear mutations in a hamster cell mutant causing a defect in complex I could be complemented by a gene on a hamster or mouse X chromosome, but not by a human X-linked gene [112]. This result was also interpreted in terms of the incompatibility of heterologous gene products.

A very informative test of genome compatibility was recently reported by Kenyon and Moraes [113]. By fusing enucleated cells from the hominoid apes (chimpanzee, pigmy chimpanzee, gorilla, and orangutan) with  $\rho^0$  (mtDNA-less) human cells these authors created "xenomitochondrial cybrids" with ape mtDNA and human nuclear

DNA. Only the combinations of human nuclei with mitochondria from the most closely related species (chimpanzee, gorilla) yielded cells capable of oxidative phosphorylation. Orangutang mitochondria in such cells did not express a functional OXPHOS system.

### 4.3 REPLICATION OF MITOCHONDRIAL DNA

Transcription and replication of mtDNA are basic processes which are closely related to both the function of the organelle and its biogenesis. The present section will deal with mtDNA replication, with an emphasis on animal mitochondria. When mitochondrial DNAs were discovered almost thirty years ago, and the first estimates of their size became available, it also became clear that the relatively small size of animal mtDNAs made them attractive candidates for study. Expectations were not disappointed, and our understanding of DNA replication and transcription in animal mitochondria is relatively far advanced. Insights from such studies will be presented first. An update on studies in other organisms will summarize the progress, provide some contrasting or unique characteristics, and emphasize some of the issues for which answers still have to be sought.

Mitochondrial DNA replication can be investigated conveniently in tissue culture, where intracellular mitochondrial proliferation is loosely coupled to cellular division and multiplication. The total number of mtDNAs has to double in each cell cycle, and therefore experimental access to the process is relatively simple in tissue culture. Replication intermediates can be found readily. Before entering into the details of this process, one should be reminded that the situation may be more complicated in an intact animal, and several aspects of the process need to be discussed separately. It is necessary distinguish between the number of mitochondria per cell and the number of mtDNA molecules per cell (estimated to be a few thousand). A priori it makes sense for mitochondrial DNA replication to be controlled to maintain a roughly constant number of mitochondria per cell, and thus there has to be a coordination with DNA replication in the nucleus. However, during embryonic development and differentiation in mammals the number of mitochondria per cell may be altered, depending on the energy requirements of particular tissues. It is possible that in adult tissue such as muscle and brain there is also turnover of mitochondria and hence mtDNA replication which is not linked to nuclear DNA replication and cell division. A particularly fascinating situation appears to occur during oogenesis, where only a small subset of the total population of mtDNA molecules is involved in mtDNA replication to generate the large number of mitochondria found in the mammalian egg. While there is as yet no proof of this hypothesis, such a "bottleneck" has to be invoked to explain observations on mitochondrial inheritance and the phenotypic expression of mitochondrial mutations in heteroplasmic cells [114]. A more complete discussion of this topic will be found in Chapter 7.

In cells in tissue culture mtDNA replication seems to be loosely coupled to nuclear DNA replication, with the precise mechanism of the control of copy numbers yet to be elucidated. On the other hand, the process of replication itself is quite well under-

stood [115–117]. Mammalian mtDNAs, and probably most vertebrate mtDNAs, have two origins of DNA replication, one responsible for each strand. The asymmetric distribution of nucleotides (G + C) in mammalian mtDNA allows one to distinguish a “heavy” and “light” strand on alkaline CsCl gradients, and hence one can also speak of  $O_H$  as the origin of H-strand synthesis, and  $O_L$  as the origin of L-strand synthesis.  $O_H$  is located within the region devoid of genes referred to as the displacement loop, or D-loop region, and at other times as the control region. Initiation and elongation of the H strand is the first event, and it proceeds a considerable distance around the circle; only after the second origin ( $O_L$ ) is displaced as a single-stranded template will DNA replication of the other strand and in the opposite direction begin.  $O_H$  is therefore dominant, and  $O_L$  is not an independent origin, since DNA replication beginning at this origin without the prior activation of  $O_H$  has never been observed. It is significant that  $O_L$  is located within a cluster of five tRNA genes. When this region has been displaced and is found in single-stranded form, it is likely that a secondary structure is formed which contributes to the functioning of the origin. Support for such a hypothesis comes from the observation that in chickens the  $O_L$  sequence itself (found in mammals) is absent, but a similar cluster of five tRNAs is still found at this location [118].

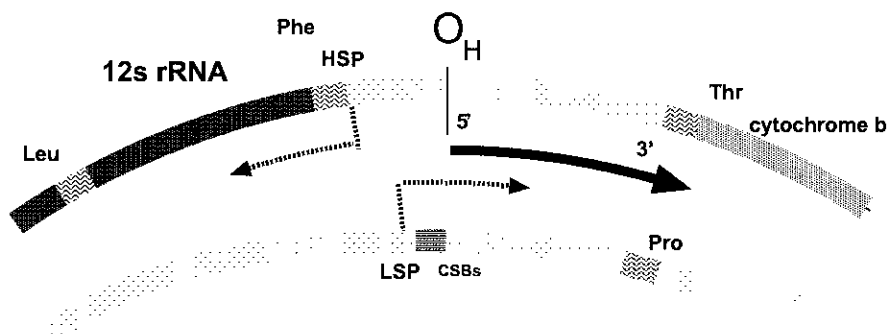
Strand elongation by a mitochondrial DNA polymerase (polymerase  $\gamma$ ) proceeds in the usual 5'  $\rightarrow$  3' direction. The nature of this polymerase and its relationship to the various bacterial and eukaryotic DNA polymerases is an interesting one [see Reference 47 for a more explicit discussion]. Its low abundance has made biochemical studies a challenge. Pol  $\gamma$  prefers ribohomopolymer templates, and therefore it was initially thought to represent a cellular reverse transcriptase resembling the reverse transcriptase of tumor viruses. Later it was recognized that nuclear preparations had been contaminated with mitochondria. It is antigenically completely different from the viral enzymes, and is further distinguished by its inability to use natural RNAs as templates. Other distinguishing characteristics include stimulation by salt, resistance to aphidocolin, inhibition by *N*-ethylmaleimide, and by dideoxynucleotide triphosphates [47].

The most highly purified preparations suggested that it exists of a holoenzyme of ~125–140 kDa, and it may be associated with a smaller subunit (35–54 kDa) to which the function of a 3' to 5' exonuclease and proofreading enzyme had been attributed. When pol  $\gamma$  genes were cloned (e.g., MIP1 from *S. cerevisiae*), a 140-kDa polypeptide was found to include both polymerase and exonuclease domains with recognizable homology to prokaryotic, A-type DNA polymerases, for example, *E. coli* DNA polymerase I. The cloning of *X. laevis* and *D. melanogaster* pol  $\gamma$  genes confirmed the conclusion that the polymerization and 3' to 5' exonuclease functions are combined in one polypeptide [117]. The role of the smaller subunit and other accessory factors needed for replication remains to be clarified.

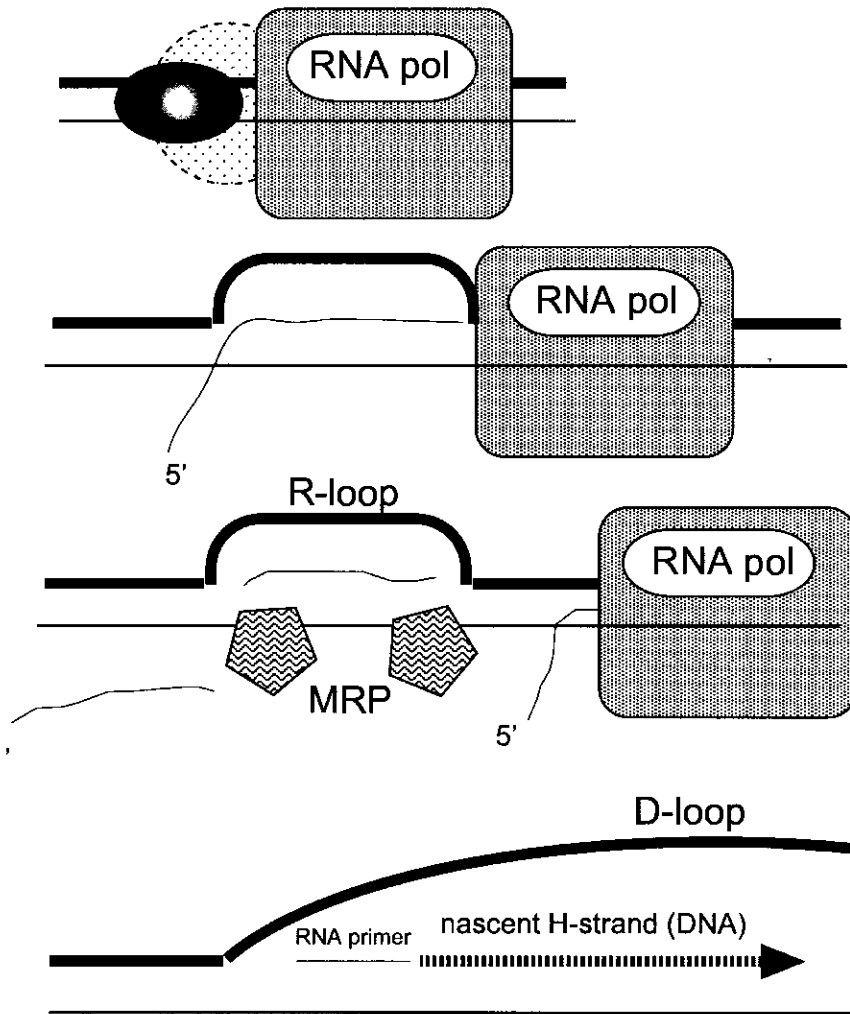
Mechanistically, however, there are no novel or unusual aspects of the mechanism of DNA strand-elongation in mitochondria. The same cannot be said about priming and initiation of DNA replication. It became clear some time ago that DNA replication and transcription of mtDNA were directly and intimately linked. The first indication was that the two known promoters for transcription are both located in the

control region. They are ~150 bp apart in mouse and human mtDNA and control transcription in opposite directions; hence they can be referred to as the light-strand promoter (LSP) and the heavy-strand promoter (HSP). Their precise identification became possible with the establishment of a faithful *in vitro* system for transcription. Three key findings were made when detailed characterizations were made of the major nascent DNA chains at the earliest stages in DNA replication, and of all the transcripts from the LSP. First, all newly initiated DNA chains had a 5' end which corresponded to the sequence ~200 nt downstream from the LSP. Second, LSP transcripts were of two types: the expected long transcripts which would later be processed to yield eight tRNAs and one mRNA, and short transcripts with 3' termini precisely or closely adjacent to the 5' termini of the nascent DNA chains. It was a very plausible jump to the conclusion that the RNA transcripts might serve as primers for the start of DNA replication from the  $O_H$  origin. A satisfying confirmation of this idea was provided by the third finding: LSP transcripts still covalently attached to the nascent DNA (in the mouse). Transcriptional activation is therefore closely linked to and a prerequisite for leading strand DNA replication. A current model [114] incorporating many findings is presented in Figures 4.5 and 4.6.

Figure 4.5 shows the control region of the vertebrate mt genome between the Phe-tRNA on the left and the Thr-tRNA on the right. Initiation of transcription at the L strand promoter generates an RNA which remains firmly associated with the DNA in the region defined by the conserved sequence blocks (CSBs), displacing the DNA strand to form a loop. Subsequent processing on either side releases a long downstream transcript and a short upstream fragment. The RNA segment remaining in the R loop can then serve as primer for DNA synthesis. The D loop is formed when the extension of the newly synthesized H strand is arrested (see below). Initiation of mtDNA replication in other vertebrates is likely to be quite similar, with some variations in the size of the control region, the nature of the CSB elements (with CSB1 apparently the most essential), and the spacing between them [117].



**Figure 4.5** The D-loop region of mammalian mitochondrial DNA and the flanking genes. HSP and LSP are the heavy-strand and light-strand promoters. A processed transcript from the LSP serves as the primer for DNA replication from the  $O_H$  origin. (Modified after D. Clayton. [115,116])



**Figure 4.6** Detailed schematic representation of the generation of the RNA primer for DNA replication. RNA polymerase and factor(s) generate an RNA transcript. This transcript is modified by a mitochondrial RNA processing enzyme (MRP), leaving a short RNA tightly annealed to the DNA and creating an RNA loop. Priming from this RNA of DNA replication leads to the generation of the larger D loop. (Modified after D. Clayton [115,116].)

Recent studies *in vitro* have provided additional support to this model [119]. RNA of defined length made *in vitro* was capable of annealing to supercoiled mouse mtDNA under defined conditions, creating an “R loop.” This R loop had been previously observed in *in vitro* transcription experiments with purified mitochondrial RNA polymerase activity, and it had been shown to consist of a “persistent” RNA-DNA hy-

brid. It was also possible to create the R loop by in vitro transcription with SP6 polymerase from an SP6 promoter inserted into mouse mtDNA control region. Its formation was not dependent on the presence of a particular RNA polymerase or additional protein, but did require negative superhelical turns in the mtDNA.

The R loop with the RNA-DNA hybrid must have unusual stability, since it is stable to manipulation and purification without branch migration, and even linear restriction fragments containing the R loop are stable for considerable time at 37°C without loss of one of the polynucleotide strands. The RNA in the R loop is not base paired with the complementary DNA strand along its entire length. From a detailed examination and comparison of RNase H, and RNase T1 cleavage sites in the R loop with those of a double stranded hybrid made from the same RNA and its cDNA it was concluded that a unique structure must be formed. A model of the R-loop has emerged in which intramolecular RNA-RNA interactions stabilize and protect distinct regions against single strand-specific nucleases (stem-loops?), while other segments interact with unique sites on the DNA. An examination of the relevant DNA sequence has revealed three conserved sequence blocks (CSB I, CSB II, CSB III), and both CSB II and CSB III contain poly G tracts and are absolutely required for R-loop formation. From the results of the probing with single strand-specific nucleases and RNase H the whole structure cannot be represented by conventional Watson-Crick base-pairing schemes. The CSBs sequences attracted attention as potential cis-acting regulatory regions as early as 1981, because they were short, highly conserved regions within an otherwise highly divergent region [115,117].

Endonucleolytic processing of the RNA in this hybrid structure is postulated to create a primer which is positioned in the correct position for extension by deoxyribonucleotides with DNA polymerase. A candidate enzyme performing this function was isolated by Clayton's laboratory in 1987 [116,120,121]. It was shown to be a site-specific endonuclease capable of cleaving RNA transcripts from the D-loop region between CSB II and CSB III, and most intriguingly, this RNase MRP (mitochondrial RNA processing) contains an RNA component encoded by nuclear genes in mouse and humans. Base-pairing interactions between this RNA and the substrate RNA for this nuclease are therefore likely to guide substrate selection and cleavage site selection. Undoubtedly, the secondary structure of the LSP transcript within the R loop is also a strong determinant of specificity and activity. Identical ribonucleoproteins have been isolated from several mammals, the frog *Xenopus laevis*, and even yeast. Cloning of these genes as well as the genes encoding the RNA component has confirmed a high degree of evolutionary conservation [117]. As discussed in the most recent review by Clayton [117], it was a challenge to show that the MRP is indeed found in mitochondria, since a very similar activity is also found in the nucleus where it is involved in RNA processing. Two types of experiments now support the conclusion that there are two pools of MRP: 1) MRP RNA was localized in mitochondria by in situ hybridization experiments; and 2) mutations in the yeast MRP RNA gene have been shown to cause a "mitochondrial phenotype."

Finally, one must consider that the stability of the R loop creates a new problem at the completion of the heavy DNA strand synthesis. Before the ends can be ligated to form a covalently closed circle, the R-loop structure has to be removed. The existence

of RNase H-like activities found in submitochondrial protein fractions suggests candidates for the removal of the primer, since mitochondrial DNA polymerase does not have an associated RNase H activity [47].

L-strand synthesis beginning at  $O_L$  is also primed by an RNA primer complementary to a T-rich segment of  $O_L$  flanked by tRNA genes. The T-rich segment has the potential for stem loop formation, once it is dissociated as a single-stranded DNA. At a location close to the base of the stem a switch occurs from ribonucleotide incorporation to deoxyribonucleotide incorporation by DNA polymerase. The priming RNA is thought to be synthesized by a mtDNA primase which recognizes  $O_L$ . An activity with such a capacity has been described [122], but much needs to be learned about the recognition of the origin, the limited synthesis of the primer, and the ultimate cleavage of the primer from the L-strand. Since the transition from RNA to DNA is localized within a sequence which is normally transcribed as a complete tRNA within a polycistronic transcript, it is possible that the activity/mechanism for splicing out the tRNA performs a dual role in removing the RNA primer from the L strand.

A curious and still unresolved problem is related to the existence and persistence of the D loop and its function [117]. The D loop is a bubble in which one strand of the control region has been copied and the other has been displaced. It includes the ~1 kb control region of mammalian mtDNA. The exact size of the D loop is species-specific. Near the 3' end of the nascent (arrested) strand conserved termination-associated sequences (TASSs) have been identified in vertebrates, and the existence of a specific DNA binding activity has been proposed from experiments with bovine mitochondrial extracts [123]. It is not yet known whether D-loop strands are simply elongated when replication proceeds, or whether there is a distinct cycle of initiation through the D-loop region. The discrete size of the D loop indicates that there is a pause in strand synthesis, and overcoming this arrest may be another mode of control. It has also been suggested that it plays a role in transcriptional regulation or mtDNA segregation.

It has been noted that whereas gene order in animal mtDNA is quite stable over long evolutionary periods, the nucleotide sequences of individual genes differ markedly between species, and a significant sequence heterogeneity (polymorphisms) is found even among human populations, a fact which is of great interest to evolutionary anthropology and in forensic applications (see Chapter 8). Of even greater potential interest is the current hypothesis that an accumulation of mutations in mtDNA is contributing to senescence in general, and perhaps specifically to the symptoms of patients suffering from Parkinsonism and other neurodegenerative diseases (see Chapter 7). A standard interpretation is that either DNA replication in animals is exceptionally error prone, and/or there is no or only a limited capacity for DNA repair such as mismatch repair or removal of thymine dimers. An error rate near 1 per million nucleotides incorporated is relatively low, thanks to the 3' to 5' exonuclease activity [47]. The capacity for mtDNA repair has been examined by a number of authors, with damage induced with a variety of agents. A recent review summarizes the current status of this field [117], and a further discussion will be deferred to Chapter 7.

There is only limited evidence for recombination between mammalian mtDNAs, in part because the process is difficult to study due to the strictly maternal inheritance of mtDNA (see Chapter 7). More recently, some generalizations about the absence of recombination and repair mechanisms have been challenged [124]. Since a genetic approach is still out of reach for the study of mammalian mitochondria, biochemical assays were made with mitochondrial extracts to look for activities which could carry out recombinational repair on genetically engineered substrates *in vitro*. Since the presence of a "robust" homologous repair activity was demonstrable in liver mitochondria, the authors argued that this mitochondrial mechanism was exclusively used for DNA repair through gene conversion. The previous failures to observe genetic recombination were due to the failure to detect crossovers.

It is generally stated that mtDNA replication is not tightly coupled to nuclear DNA replication during the S-phase of the cell cycle. Some control is undoubtedly exercised through the availability of deoxyribonucleotides in the cell, and hence through the activity of ribonucleotide reductase. How the import of such deoxyribonucleotides into mitochondria is achieved and whether pool size in the matrix is different from cytosolic/nuclear pools are unanswered questions at this time. Since the copy number of mtDNA per cell remains constant in tissue culture, there must be some check on mtDNA replication, and likely sites of control involve initiation at the origin in the D loop ( $O_H$ ). In the current model transcription from the LSP is a prerequisite, but a rate limiting step could still be at the level of the RNase MRP which creates the RNA primer. In mouse L cells lacking cytoplasmic thymidine kinase, but having a mitochondrial thymidine kinase, mtDNA replication is conveniently studied by incorporation of radioactive thymidine, or of bromodeoxyuridine. Such studies have revealed that mtDNAs are apparently selected at random, with some being replicated twice during the cell cycle while others are not replicated at all. Organelle genomes like mtDNA (and chloroplast DNA) have been referred to as "relaxed genomes," and both their replication and their segregation can be considered to be relaxed [125]. This behaviour leads to their general failure to obey Mendel's laws: alleles can segregate during mitosis as well as during meiosis, inheritance is often uniparental, and there is the possibility of intracellular selection of advantageous alleles among hundreds if not thousands of copies of the genome.

A recent very interesting study by Davis and Clayton [126] has revisited the issue of mtDNA replication in mammalian cells, taking advantage of laser-scanning confocal microscopy to detect newly incorporated BrdU into mtDNA. The total population of mitochondria within a cell was viewed by dye-labeling (using Mitotracker™), and BrdU incorporation was detected by immunocytochemistry. When short labeling periods were used (1–2 hours), BrdU incorporation occurred preferentially into perinuclear mitochondria, and distal mitochondria could be stained with antibody only after prolonged labeling. Since mtDNA was present in all mitochondria (determined by ethidium bromide staining), mtDNA replication must occur preferentially in the vicinity of the nucleus. The dependence on the nucleus was further demonstrated by experiments with enucleated HeLa cells. In such cytoplasts mitochondrial DNA replication was completely arrested (even though mi-



tochondria looked normal and retained their membrane potential). Similarly, in human platelets no BrdU was incorporated into mtDNA. Thus, a nucleus is not only required, but factors provided by the nucleus are apparently not freely diffusible throughout the cell. On the other hand, it is not necessary to have ongoing DNA replication in the nucleus. This was shown most clearly with PC12 cells induced by NGF to differentiate into neuronlike cells with a cell body and long extensions (neurites) and axons. Mitochondria in the cell body (perinuclear) incorporated BrdU within a period of a few hours, while mitochondria in the neurites and growth cones were not labeled after such relatively short labeling periods. With time, labeled mitochondria appeared in the periphery, and the authors speculated that they had been transported there by axonal transport. Inhibiting DNA replication with the chain terminator dideoxy cytidine (ddC) while still allowing some BrdU incorporation suggested that such mitochondria (with unfinished replication intermediates) remain in the cell body, that is, are not subject to axonal transport toward the periphery.

The interpretation of these provocative results is highly speculative at this time. The authors suggest that either some type of tethering to a cytoskeletal perinuclear element is required, or that an essential component of the mtDNA replication machinery must be restricted to the perinuclear region of the cell. The nature of the required factor is not further specified. A nuclear-encoded protein would have to be synthesized within this restricted domain of the cell, or concentrated there before import into the mitochondria. It is also possible that the synthesis of deoxynucleoside triphosphates is limited to this region of the cells, although one would expect such small molecules to diffuse rapidly throughout the cell.

The RNA and DNA polymerases as well as all of the nucleases, maturases, transcription factors, repair enzymes and various DNA binding proteins are encoded by nuclear genes, and ultimate control of their expression is exerted in the nucleus. A full discussion of the nuclear genes relevant for mitochondrial biogenesis and their expression is presented in Section 4.2. Whether or not the transcriptional activator mtTFA acts as the master switch (in the earlier literature it had been referred to as MtTF1) is still an open question [117]. Curiously, it does not bind exclusively at the two promoters (LSP and HSP), but also within the region of the CSBs where it may interact with the RNase MRP and control primer production.

Mitochondrial DNA replication in most nonvertebrate organisms remains to be explored. The yeast, *S. cerevisiae*, has as expected, served as another model system with the advantage of the exploitation of genetic approaches. Thus, the pol  $\gamma$  gene (*MIP1*) was first isolated and characterized from yeast [52], two mitochondrial transcription factors (sc-mtTFA and sc-mtTFB) have been defined, and the gene for the RNA component of the RNase MRP gene (*NME1*) has been cloned. However, the size of the yeast mtDNA has made the characterization of replication intermediates and the identification of replication origins difficult. The same problem is even more acute with the still larger plant mtDNAs.

Now that it is established that many plant and fungal mtDNAs are linear molecules [11], and that terminal sequences may have a telomere-like function, understanding mtDNA replication includes another challenge: how are linear 5' ends dealt with by

the replication machinery. The search for proteins active at the ends is on, and some initial success in *Candida parapsilosis* has been reported [11].

## 4.4 TRANSCRIPTION OF MITOCHONDRIAL DNA-RNA METABOLISM

### 4.4.1 Transcription in Mammalian Mitochondria

In the previous section on mtDNA replication reference has already been made to transcription and the role it plays in providing the primers for DNA replication in mammalian mitochondria. Discussion will again focus first on the mammalian system because of its relative simplicity and the advanced level of understanding that has been reached in this system [17,116].

One needs to be reminded first of the absolute compactness with which genes are arranged on the mammalian mt genome. From the first complete sequences it became apparent that there was virtually no room for promoter elements between genes, and the choice was between having very few promoters from which large segments of the mtDNA were transcribed, or postulating promoters which overlapped the transcribed and coding sequences of the various genes. Pioneering experiments in the laboratories of Clayton and Attardi and others came to converging conclusions and interpretations.

In a chronological order which was dependent on available technology, the first relevant experiments addressed the question: Is the mitochondrial genome transcribed? Labeling with [<sup>3</sup>H]uridine and cell fractionation studies gave the earliest indications that rapidly labeled RNA was associated with a membrane fraction which included mitochondria [17]. MtDNA had played a crucial part in the development of methodology employing CsCl gradients in the ultracentrifuge, and the subsequent discovery of the phenomenon of DNA supercoiling with the help of the intercalating dye ethidium bromide. As a result, relatively pure preparations of mtDNA were available when RNA-DNA hybridizations were first exploited to study gene content, some years before sequencing and cloning became the tools for high resolution analysis. Such studies gave the first indications that most sequences of mtDNA in HeLa cells were transcribed [17]. Another question raised immediately was: How many peptides are made in mitochondria? The capacity for autonomous protein synthesis was strongly suggested by the discovery of ribosomes in mitochondria, although their properties differed quite significantly from bacterial and eukaryotic cytoplasmic ribosomes. Using isolated mitochondria, or whole cells in which cytoplasmic protein synthesis was inhibited by cycloheximide, it could be shown by labeling with [<sup>35</sup>S]methionine and electrophoretic fractionation that a small number of peptides was synthesized in mitochondria, and eventually all peptides predicted from the primary sequence could be accounted for on gels. Later, as cloned probes for subregions of mtDNA became available, Northern analyses and S1 protection analyses were used to establish that each peptide was encoded by its own distinct mRNA, but very large molecules were also seen which could be identified as presumptive precursors by pulse-chase experiments. From such studies the consensus emerged that a few relatively large transcripts from both strands must be made which are subsequently

spliced and processed to create individual mRNAs, rRNAs, and tRNAs. It is now accepted that the transcription units include the entire H strand and the entire L strand. Having the entire mtDNA sequence from humans and cows was clearly of enormous help in the interpretation. In this regard it is worth noting that mtDNA sequencing and the characterization of mature mtRNAs were carried out simultaneously in Cambridge and at Cal Tech, with an ongoing exchange of complementary information. The papers on the human mtDNA sequence and on the precise mapping of the transcripts were published in the same issue of *Nature* [6,127,128]. A very broad and authoritative review from a historical perspective has been written by one of the pioneers and major contributors to this accumulated knowledge, G. Attardi [17]. Not the least of the technical problems to be solved in the course of these studies was to culture an estimated kilogram of HeLa cells.

The existence of the D loop identified by electron microscopy, and by the finding of a ~1 kb region free of coding information between two tRNA genes, drew attention to this region as a possible control region, but in the early 1980s there were no criteria for the identification of a mitochondrial promoter, and no procedures existed then or now to test promoters with reporter genes *in vivo* in mitochondria. A breakthrough was achieved in 1983 when the first *in vitro* system for initiating transcription from mtDNA in a human mitochondrial extract was described [129]. Transcription starting specifically from the D-loop region was observed, leading in follow-up experiments to the identification of the L-strand and H-strand promoter elements (LSP and HSP) in the D-loop region. For clarification it may be necessary to state that the D loop identifies the control region (D-loop region), but the D loop itself (the bubble created by L-strand displacement by a short DNA strand) is somewhat shorter than the entire region. A systematic destruction of these elements by the insertion of small linkers (linker-scan analysis across the entire region) confirmed their importance and their limited extension within the control region. The two promoters are approximately 150 bp apart, but do not overlap and function independently. Upon further analysis it was confirmed that L-strand transcripts are giant polycistronic transcripts containing the mRNA sequence for the NAD6 subunit and eight tRNAs. However, transcription in the opposite direction from HSP starts at two closely spaced initiation sites with differing activity. One transcript contains the tRNA<sup>Phe</sup> and tRNA<sup>Val</sup> genes and the rRNA genes, terminating at the 3' end of the 16S rRNA. The second, overlapping transcript is another giant polycistronic RNA which is processed to yield the other mRNAs, and most of the tRNAs encoded by the H-strand. The higher rate of initiation and the termination at the 3' end of the 16S rRNA assures that the rRNA species are synthesized in much greater abundance (15- to 50-fold) than the mRNAs.

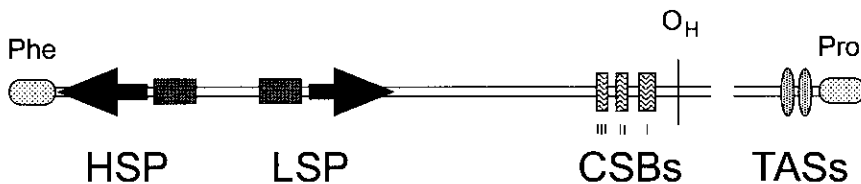
The D-loop region is functionally conserved in all vertebrates, but the nucleotide sequences of the different elements themselves are not conserved. Because of this lack of sequence conservation it is also less clear whether all vertebrates (mammals?) contain all of the elements that are found in the human D loop. However, it is flanked in almost all cases by the tRNA<sup>Phe</sup> downstream from the HSP (Figure 4.5), and by a tRNA<sup>Pro</sup> downstream from the LSP (Figure 4.5). An immediately adjacent gene is the 12S rRNA gene. Since the tRNA and rRNA sequences are highly conserved in vertebrates, these flanking genes can provide (degenerate) primer sequences for use in

PCR amplification, and D-loop regions from other vertebrates can be conveniently amplified and cloned for further analysis. The detailed description of the human and mouse D-loops (Figure 4.7) will serve as examples, illustrating common features, but it should be emphasized that D loops in different vertebrates vary in size, in sequence and even in the number of distinct promoters they contain. In chicken and frog (*X. laevis*) D loops are small, and promoters may overlap, making them appear to be bi-directional promoters. There may be multiple transcription initiation sites in some vertebrates, and some promoters (human, mouse) have additional cis-acting elements for tuning promoter activity.

The stage was set for a further fractionation of mammalian mitochondrial extracts, which yielded two significant protein components: a mtRNA polymerase and the mitochondrial transcription factor now referred to as mtTFA [66]. No other significant factors have been purified, although there are other DNA binding proteins whose role in transcription and transcriptional regulation may be subtle. Binding of mtTFA to the D-loop DNA could be demonstrated in the absence of mtRNA polymerase, confirming a direct interaction of the factor with the DNA. This conclusion was corroborated and extended by footprinting and additional linker-scan analyses. MtTFA bound upstream of each of the two transcription start sites (defined by nucleotides from positions -10 to -40), but surprisingly, it also bound to a region between the conserved sequence boxes I and II (CSB I and CSB II) which play a role in the generation of the RNA primer for H-strand synthesis. The affinity of each promoter for mtTFA is different: the LSP is favored, consistent with its greater activity in vitro. It is probably also significant that the LSP controls primer synthesis for DNA replication. When the two sequences at each promoter identified by the footprinting are compared, homology emerged only when one sequence was inverted with respect to the orientation of the promoter. In other words, if this homology is significant, it implies that mtTFA can function in a bi-directional manner.

Again, generalizations from the discoveries in human or mouse mitochondria to other vertebrates must be made with caution, since promoters differ significantly in nucleotide sequence, and the number and spacing of critical elements. There are indications that MtTFA binds to mtDNA at other sites, raising the possibility of other functions being performed by this factor. So far it has been isolated from humans, the mouse, from the frog, *X. laevis*, and from yeast (see below).

The cDNA for this protein has been cloned, and the basic structural features of the peptide have been deduced from protein sequence comparisons. A mitochondrial



**Figure 4.7** A detailed view of the promoter regions of mammalian mitochondrial DNA. (After D. Clayton [115,116].)

targeting sequence of 42 amino acids is presumably cleaved off, leaving a mature protein with 204 amino acids. The most notable features are two segments of ~70 amino acids recognized to be present also in high mobility group proteins (HMG) of the nucleus. These segments are likely to play a role in DNA binding, although the mechanism of transcriptional activation is not obvious. There are 31 residues separating HMG Box 1 from HMG Box 2, and very short flanking regions at the N-terminal and C-terminal, respectively.

A comparison of the human and mouse control region and experiments in both systems had confirmed the functional significance of this region in DNA replication and transcription and had revealed a similar arrangement of the relevant sequence elements. Unexpectedly, there was a notable divergence in nucleotide sequence, explaining, perhaps, why human mitochondrial extracts failed to transcribe mouse mtDNA and vice versa. When the mtRNA polymerase and the mtTFA from each species were sufficiently well purified, it became possible to set up mixed assays to determine whether the species specificity was entirely attributable to the recognition of the mtDNA by mtTFA. It was anticipated that heterologous polymerases would have comparable activities in such assays, but on the contrary, it was found that the mtTFA can function on a heterologous template. Thus, species-specific transcription is due to a subtle difference in the polymerases, or due to as yet unrecognized and unidentified factors in the extract or in the partially purified fractions.

#### 4.4.2 Transcription of mtDNA in the Yeast *Saccharomyces cerevisiae*

A comparison of the structure of the yeast mt genome with that of animals immediately suggests that many more transcriptional start sites must exist, since the genome is about five times larger, the genes are dispersed, and AT-rich, noncoding sequences are inserted between them. At least 13 transcription initiation sites have been mapped, and there are an additional four sites for the synthesis of primers to be used in DNA replication [131,132]. The simple promoters all have a 9 bp consensus sequence: 5'-ATATAAGTA(+1)-3'. The last A also corresponds to the 5' end of the transcript. Like in animal cells, polycistronic RNAs (but smaller) are still produced which must be cleaved and processed to yield mRNAs encoding peptides, tRNAs and rRNAs. A total of seven peptides are encoded by the mRNAs: cytochrome oxidase subunits I, II, III (COXI, COXII, COXIII), apocytochrome b (COB), subunits 6, 8, and 9 of the  $F_0$  complex of the mitochondrial ATP synthase, and a ribosomal protein VAR1. There are some nonessential peptides encoded by introns of COXI, COB, and the 21S rRNA gene which are required when the introns are present (maturases), or can be active in the transposition of the introns, but in intronless strains they are absent and not missed. Three open reading frames have so far been identified only from sequencing, not from isolation of transcripts or from the discovery of mutations within these sequences. It has been speculated that such as yet unknown functions may be required for DNA replication or general recombination [133].

Transcription itself is accomplished by a core RNA polymerase (145-kDa, encoded by the nuclear *RPO41* gene), and a specificity factor (43-kDa, encoded by the nuclear *MTF1* gene). Both genes have been cloned and the proteins have been char-

acterized [134]. The polymerase bears a relationship with polymerases of the bacteriophages T3 and T7, while the specificity factor resembles the bacterial sigma ( $\sigma$ ) factors, but it has also been compared to the MtTFA protein in mammalian mitochondria. The Mtf1 protein is released from the transcriptional complex after the first few nucleotides have been polymerized and is then re-cycled. Its function is primarily to assure specificity, stimulating polymerase activity only a few fold. When the MTF1 gene is deleted, the maintenance of the yeast mt genome is no longer assured.

#### 4.4.3 Transcription of mtDNA in Plant Mitochondria

An up-to-date and expert review of the present state of knowledge has recently been written by Binder, Marchfelder, and Berennicke [135], and references to much of the primary literature can be found in this review. The area is in an explosive state of growth, and many examples in the literature on the one hand permit increasingly reliable generalizations on certain aspects, while on the other hand plant specific differences are also apparent [18]. For example, monocot and dicot plants share many broad features, but may differ in detail. A distinction for all plants is the simultaneous presence of chloroplasts. Mitochondria and chloroplasts are often discussed together, not only because of obvious similarities, for example, the observation of RNA editing in both plastids, but because it is likely that factors may be shared between these two organelles.

The variable size of plant mtDNAs has already been emphasized. At the same time, a large fraction of this DNA does not have any obvious coding or regulatory function, and generally the same limited number of proteins, rRNAs and tRNAs is encoded by plant mtDNA as in the mtDNA of animals and fungi. Only a limited number of multicistronic transcription units have been described so far, and these may be more common in plant mitochondria having relatively small genomes with a denser spacing of genes. As described earlier, the intergenic regions of plant mtDNA can be expanded or contracted by frequent recombination events, which can also rearrange the position of genes relative to each other.

Many examples of monocistronic transcripts encoding proteins (atp6, atp9, cytb, cox1, cox2, and cox3) have been described. Such transcripts include extended untranslated regions at the 5' and 3' end, frequently hundreds of nucleotides in length. In contrast to other species, plant mitochondrial transcripts are not polyadenylated. Since the primary 5' ends of such transcripts are not capped, they can be capped by an *in vitro* reaction, which subsequently permits a distinction between a 5' end of a primary transcript and the 5' end of a mature mRNA generated from RNA processing. By this method, at least 15 primary transcripts (and promoters) have been identified in *Oenothera* [135], with the promoters distributed over the entire genome. The analysis of primary transcripts can be complicated by the existence of multiple promoters, spaced over several hundred nucleotides upstream of the coding sequence. Multiple transcripts of different size may therefore be found. It has not yet been resolved whether such arrangements have a regulatory significance in differentiation and development. Another potential complication is the possibility of multiple copies of a gene in the genome which may yield transcripts with differing 5' and/or 3' UTRs.

A detailed analysis of several promoters has been greatly facilitated by the development of *in vitro* transcription systems, permitting the examination of promoters and derived variants with deletions, substitutions, and linker-insertions. From such an approach a promoter region of about 17 nucleotides has been identified in monocot plant mitochondria (wheat, maize) which includes a conserved tetranucleotide CRTA at the transcription initiation site, and a conserved purine-rich stretch about 15 nt upstream of the transcription initiation site. In dicots (e.g., pea), the CRTA motif is also conserved, and an A-rich region is found upstream which is important as demonstrated by *in vitro* mutagenesis experiments. *In vitro* studies also permit the testing of a promoter from one species with extracts from another species, and by such means functioning promoter sequences can be confirmed, but differences in efficiencies are indicative of differences in the interaction of promoters with species specific factors. Such differences cannot be deduced from simple inspection of primary sequences.

Not all promoters are identical on a specific mtDNA. Ribosomal RNAs are apparently transcribed from promoters with a distinct sequence in both monocots and dicots, based on sequence inspections or on *in vitro* experiments with homologous or heterologous mitochondrial lysates. Such observations have given rise to speculations about different transcriptional activators, and even about the possibility of more than one RNA polymerase in plant mitochondria, a situation encountered in chloroplasts of higher plants [136]. Differences in RNA polymerases, factors and promoter sequence suggest that activity at different promoters may be variable and regulated. Such a conclusion is supported by run-on experiments in isolated organelles or lysates, that is, elongation of pre-initiated transcripts with added nucleotides. Ribosomal RNAs and ribosomal protein genes were found to be most actively transcribed, and distinctions were found in one case between the other transcripts. Comparison of such data with the observed steady state levels of transcripts *in vivo* is clearly indicative of the existence of extensive posttranscriptional regulatory mechanisms, most likely at the level of mRNA stability.

#### 4.4.4 Transcriptional Termination

Transcription of an entire circular template in animal cells raises the question whether true transcriptional termination occurs. This has been a difficult question to answer, but a region at the 3' terminus of the rRNA gene and immediately adjacent to the tRNA<sup>Leu(UUR)</sup> gene has attracted attention based on *in vitro* experiments showing transcription termination at this site [137], and on very provocative finding of a ~34 kDa protein that can footprint this sequence. The same sequence is mutated in a patient with MELAS (myopathy, encephalopathy, lactic acidosis and stroke-like symptoms, see Chapter 7) [138], and the mutated form has a lowered affinity for this presumed termination protein [139]. A factor named mTERF (mitochondrial transcription termination factor) has recently been purified by DNA affinity chromatography using the boundary sequence between the 16S rRNA and the tRNA<sup>Leu(UUR)</sup> genes [140]. The important role of this protein is most likely related to the need for making more ribosomes than mRNAs (see above). It is not clear whether it can directly influence the rates at which the two closely spaced initiation sites are used, but by terminating the

majority of the transcripts at the boundary site the overproduction of rRNAs compared to mRNAs is assured.

#### 4.4.5 RNA Processing in Mitochondria

An equally challenging problem has been the elucidation of the precise mechanism by which the primary transcripts are processed to form individual mRNAs, rRNAs and tRNAs. It is clear that the process must recognize the interspersed tRNAs (Figure 4.8), because they are joined directly to rRNA or coding sequences. In the original publication describing the localization of the tRNA genes Attardi and his colleagues made reference to the "tRNA punctuation model" for mt RNA processing [128], an apt description of how the compact genome is organized and interpreted. In attempts to reproduce this processing *in vitro*, the primary transcript has been produced with prokaryotic RNA polymerases, but efforts to achieve processing with mitochondrial extracts have been quite unsuccessful. Thus it is possible that most of the processing occurs already with nascent transcripts.

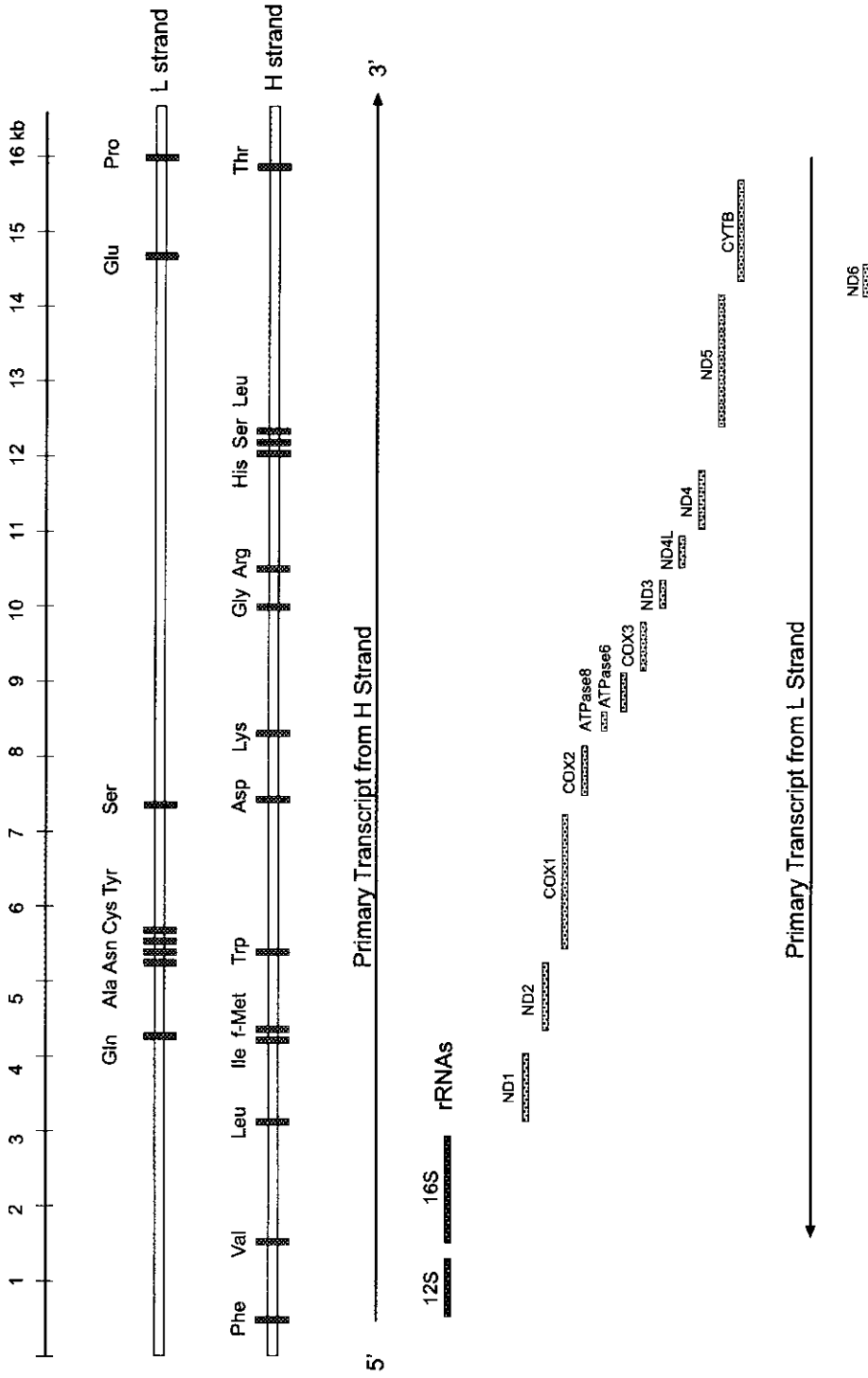
Splicing out a tRNA requires two endonucleolytic cleavages: at the 5' end and at the 3' end. Cleavage at the 5' end could occur as soon as the entire tRNA sequence has been transcribed and has assumed the appropriate secondary structure; in mitochondria this cleavage would produce the 3' end of an mRNA, for example. From the study of tRNA precursors and their processing in bacteria and other organisms, a nuclease, RNase P, has been characterized which performs the 5' cleavage, prompting a search for a corresponding activity in mitochondria of yeast and mammals which only recently has met with success [141]. Most significantly, it was found that the yeast enzyme was a ribonucleoprotein which consisted of a peptide encoded in the nucleus and an RNA encoded by the mt genome in yeast, but presumably by the nuclear genome in other organisms. The RNA has two regions highly conserved in RNAs found in other RNase Ps. They are believed to participate in base pairing interactions leading to pseudoknot formation and the formation of the catalytic core of the enzyme.

The other steps in RNA processing are even less well understood. In general, the activities of interest are present at low abundance, making purification difficult, even when a specific substrate for the assay was available from *in vitro* transcription of an engineered template. A genetic approach in yeast is a possibility, but the specific mutant would presumably have to be distinguished from a large number of other respiration-deficient mutants (*petites*).

The following types of activities remain to be purified and characterized in detail:

1. The nucleases which complete the processing of the polycistronic transcripts, for example, the cleavage at the 3' end of the tRNAs.
2. Mature mRNAs and even rRNAs are polyadenylated. Symptomatic for the extreme economy in the genome is the finding that some mRNAs do not have complete stop codons until the third adenine nucleotide is added as part of the polyadenylation reaction.
3. Fungi and plant mtDNAs encode ORFs interrupted by introns. These introns can be of type I or type II. Some of these introns include ORFs encoding mat-





**Figure 4.8** Schematic representation of the major transcripts from the  $\text{H}$  strand, and the subsequent processing to form mature rRNAs, tRNAs (not shown), and mRNAs. Only a single mRNA (ND6) and several tRNAs are derived from the transcript of the L strand.

urases or reverse transcriptase activities that play a role in intron splicing and mobility. Some of the diversity and variability of these introns in different species and even within a species have been discussed in the section on gene organization. In *Saccharomyces cerevisiae* introns have been found in the mt COXI gene and the mt COB gene. They have been characterized as self-splicing group II introns [142–144].

Processing of transcripts in plant mitochondria is certainly necessary when the transcript is polycistronic, for example, the 18S-5S-nad5 transcript in *Oenothera* mitochondria. Little is known at this time about the details of the enzymatic reactions and about the processing signals recognized by the transacting factors. 5' and 3' processing is required, depending on the transcript. For example, the 18S rRNA is transcribed from an upstream promoter and requires the removal of 25 to 120 nt, depending on the plant. Various mRNA have been found to have a double stem loop at the 3' end, and it has been hypothesized that the transcript initially extends beyond the stem loop, but is trimmed back by a 3'-exonuclease until the stabilizing secondary structure prevents further hydrolysis [135].

For the processing of tRNAs processing systems have been established in vitro from mitochondrial extracts, and such extracts have been shown to contain an RNase-P-like enzyme with an RNA moiety necessary for activity. As discussed above, this enzyme is distinct from the necessary 3'-endonuclease.

In higher plant mitochondria a number of transcripts have been found to contain group II introns that are similar to this class of introns found in yeast mitochondria. A relatively unique feature is that some introns are physically disrupted by a large intervening portion of the genome. In virtually all flowering plants such disrupted introns have been discovered in the nad1, nad2, and nad5 genes. mRNA maturation requires trans-splicing to transcripts with exons which are independently transcribed. One specific example will illustrate the surprises and unpredictability of transcription and splicing implants. The penultimate intron of the nad1 gene appears to contain an ORF potentially encoding the only maturase identified so far in plant mitochondria [145]. Trans-splicing of this intron occurs in petunia and wheat, but in the first example the maturase ORF is in the upstream half of the intron, while in wheat it is found in the downstream portion of the intron. In the broad bean this intron is not trans-spliced at all, and the maturase ORF is located between the two exons [135]. Small wonder that a review on the subject is entitled: "The mitochondrial genome: so simple yet so complex" [146].

#### 4.4.6 RNA Editing in Kinetoplastid Protozoa

One of the most bizarre and unexpected observations was the discovery of RNA editing in mitochondria of kinetoplastid protozoa. After its discovery in these organisms, editing was subsequently found in a few other organellar or nuclear transcripts in a wide range of organisms, but the extent to which it is being used in kinetoplastids is astonishing. Recently, Covello and Gray [147] pointed out that this editing appears to be completely redundant because there is no obvious reason why the same polypep-

tides could not be encoded directly by the genes and the resulting transcripts. What is editing? In the most general sense it means that an RNA, after it has been transcribed from the corresponding gene, is altered by the insertion or deletion of nucleotides which are not encoded by the gene. The unedited transcript could not be translated because there is no sensible ORF. An ORF is created only after a series of nucleotides have been inserted or deleted (or altered). A discussion of RNA editing from a perspective of an evolutionary biologist can be found in the review by Covello and Gray [147].

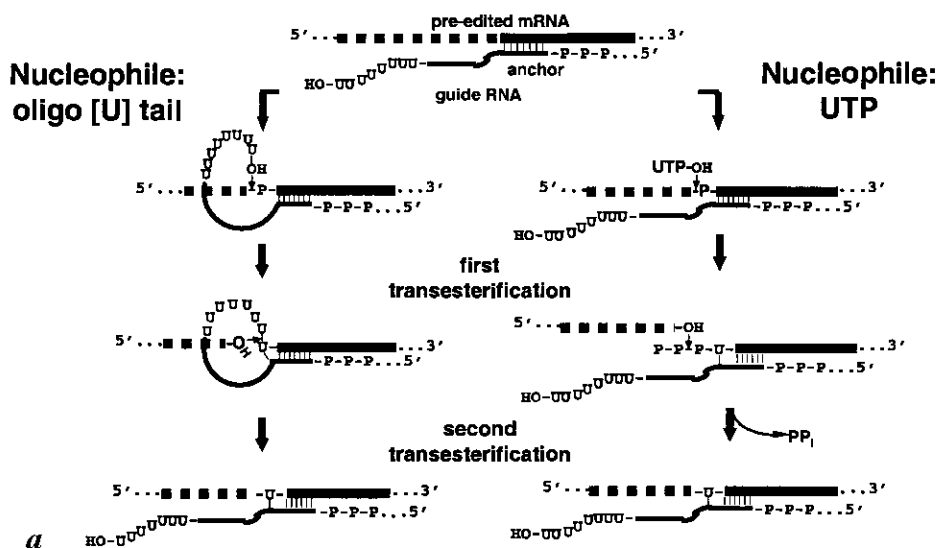
The major effort has been devoted to the study of the trypanosomatid genera *Trypanosoma* and *Leishmania*, since they are the pathogens responsible for several widespread diseases of humans and animals in tropical Africa and South America. Their interesting life cycle includes stages in both a mammalian host and insect host which serves as the vector in transmitting the pathogen.

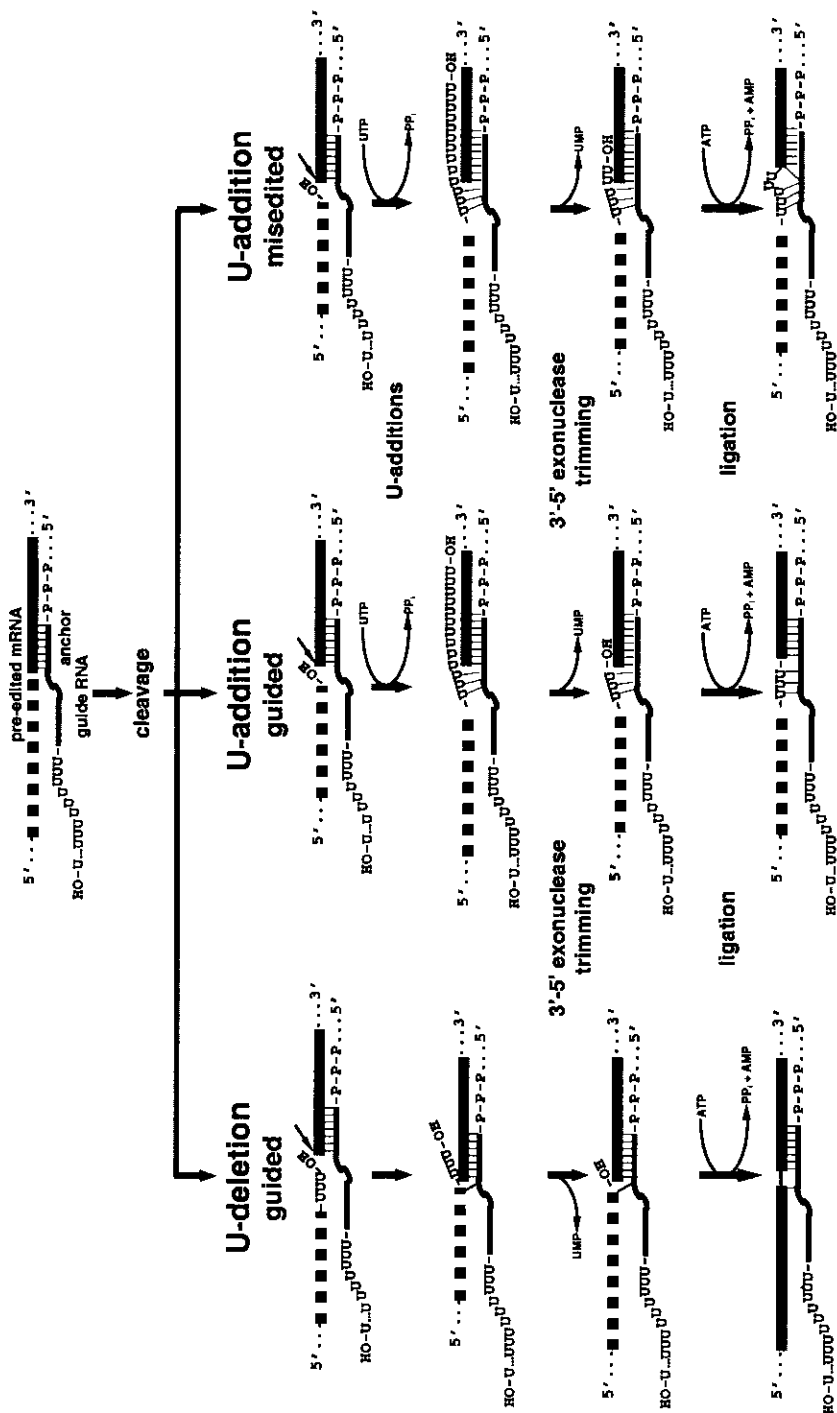
The structure of the kDNA in the kinetoplast within the single mitochondrion has been described in an earlier chapter. It remains to define and explain how mature, translatable mRNAs are produced from transcripts of the "cryptogenes" on the maxicircle. RNA editing in these organisms requires the insertion, and less frequently the deletion, of uridylyl residues, not as a rare event, but in a process involving more than half of the maxicircle transcripts. Not only are the majority of the transcripts affected, but the addition of Us is extensive, such that in some cases the transcript is doubled in size, and essential, because the addition of Us creates start and/or stop codons and the desired open reading frame. Unedited transcripts are believed not to be recognized by the translational initiation mechanism. This amazing violation of orthodoxy has been and continues to be met with questions about What? How? Why? and When? [147–149]. The first two can now be answered with some confidence and in detail. The last is a question about the evolution of this phenomenon, and answers are coming in as more and more examples become available to construct reliable phylogenetic trees [147,148]. Why? is the question most difficult to answer, and a speculative answer will be deferred until it has been made clear what is happening to the transcripts.

RNA editing is not confined to trypanosomes [150,151]. However, in mammals less than a handful of nuclear gene transcripts and some viral transcripts have been found to be edited, usually at a single position, where a C is converted to a U, or the change is U to C. In marsupial mitochondria a C to U conversion in the anticodon loop converts a tRNA<sup>Gly</sup> to tRNA<sup>Asp</sup>. The COX1 mRNA in mitochondria of the slime mold *Physarum polycephalum* is edited by a C to U conversion. In plants more extensive editing occurs in mitochondria and in chloroplasts (C to U). In distinction to the editing observed in trypanosomes, editing in the other examples involves a base change, for example, a C to U conversion in apolipoprotein B, or an A to I conversion in the AMPA receptor, by hydrolytic deamination, while editing in trypanosomes the change entails insertions and deletions of uridines. One should also distinguish clearly between editing occurring within an RNA sequence, and the "editing" which is exemplified by the polyadenylation of mRNA, or the addition of the sequence CCA at the 3' end of tRNAs. The addition of a 3' oligo(U) tail to the kinetoplastid mRNAs and gRNAs (see below) also belongs to a distinct category, requiring the activity of a mitochondrial terminal uridylyl transferase.

The editing proceeds in a systematic manner from the 3' end to the 5' end, and it requires the participation of so-called guide RNAs (gRNAs). As one portion of the mRNA becomes edited, it can base pair with another gRNA, and the editing step can be repeated further upstream in the mRNA. The gRNAs are encoded in both maxicircles and minicircles, their abundance being assured by the abundance of minicircles [41]. The complexity of the minicircles and the total number of gRNAs varies from species to species; *T. brucei* has over 300 different minicircle classes, each encoding three gRNAs. The gRNA contain sequences which are antisense to portions of the mRNA to be edited, and short "anchor" duplexes are formed between the two RNAs just 3' of the sequence to be edited. The subsequent steps can so far be modeled by several schemes involving either transesterifications, or cleavage-ligation ("cut, insert and paste," also described as the enzyme cascade model), with recent *in vitro* studies favoring a cleavage-ligation model. Each scheme still includes alternatives for the terminal reactions [151,153,154].

A model based on successive transesterification reactions is shown in Figure 4.9A. The initial reaction is a nucleophilic attack at a site specified by the guide RNA, upstream from the anchor sequence. The resulting 3' hydroxyl group on the upstream portion of the preedited mRNA can then make another nucleophilic attack leading to the insertion of only one uridine, if the initial nucleophile is UTP (scheme on the right), and may lead to the insertion of one or more uridines if the nucleophile is the 3' end of the oligo (U) tail of the guide RNA. Thus, in this model the gRNA has two functions: it serves to specify the site of insertion by the specificity of the anchor sequence, and it may provide the uridines inserted into the mRNA. While this model was initially appealing in its resemblance to RNA-catalyzed splicing reactions, and hence indicative of a potential, common evolutionary origin, recent evidence leads to a rejection of this model in favor of the cascade model shown in Figure 4.9B.





**Figure 4.9** RNA editing in trypanosomes. **A:** Double transesterification models, showing only the U insertions. **B:** The modified enzyme cascade model showing U deletion, or U addition or misedited U addition as alternatives. In the simplest version the U tail of the guide RNA is shown as a free overhang, but it is also possible that it may pair with a purine-rich sequence of the pred-edited RNA. (From reference 153, with permission.)

The anchor sequence of the guide RNA again serves to specify a point of cleavage by an endonuclease, and cleavage occurs precisely at the first mismatched nucleotide upstream of the duplex formed between the gRNA and the preedited mRNA. Uridylate residues can be added to the 3' end by a terminal transferase (addition, middle and right scheme). 3'-5' exonuclease trimming may be necessary, and may be responsible for the guided deletion of U's (left scheme) [155], and finally an RNA ligase restores the continuity in the mRNA. At this point the gRNA must be released (presumably with the help of a helicase), and the steps can be repeated at a new position.

Evidence favoring the second model is derived from significant advances in reproducing some of the required steps in vitro [reviewed in Reference 153]. In addition to a model system demonstrating the gRNA-dependent insertion of Us directly from UTP (and a requirement for exogenous UTP), it could also be shown that the required ATP is hydrolyzed between the  $\alpha$ - $\beta$  bond, as expected for an RNA ligation reaction in which AMP becomes covalently linked to an intermediate. Chemical blockage of the 3' end of the gRNA by periodation did not prevent U-insertion, eliminating this end as a potential nucleophile. As discussed in more detail by Alfonzo and coworkers [153], a major modification of the original enzyme-cascade model includes the addition of multiple U's to the 5' cleavage fragment, followed by trimming by a 3' exonuclease. This model can thus accommodate additions, deletions and even misediting in a single mechanism.

Fractionations of mitochondrial extracts from *T. brucei* and *L. tarentolae*, and the reconstitution of in vitro systems performing progressive editing at multiple sites remain major challenges of the future. Some of the relevant enzyme activities have been reported (terminal uridyl transferase, endoribonuclease, RNA ligase). These activities and mRNA and gRNA appear to be assembled in a multiprotein complex; glycerol gradient sedimentation has yielded a series of often heterodisperse complexes, depending on the species examined. The emerging view [153] is that an editing complex is formed which includes not only the postulated enzymes (and an RNA helicase), but may also include one or more structural factors required for the assembly and stabilization of the complex.

The evolutionary history and the biological significance of the phenomenon should perhaps not be discussed as dissociated topics. The extensive "pan-editing" described here has been found so far only in kinetoplastid protozoa which are representative of the earliest divergence of eukaryotic cells containing mitochondria. When trans-esterification was still a viable model, speculations that "editing and splicing have a common origin in an RNA world" were defensible, but the currently favored model exhibits little resemblance to RNA splicing, and pan-editing may have an independent origin. Editing may become superfluous in the evolution of species when edited RNAs can replace the cryptogenes by a mechanism termed *retroposition* [148], unless there is continuous selective pressure to maintain it. In this context it is interesting that continuous culturing of a *Leishmania tarentolae* strain in the laboratory was observed to have lost minicircles, and hence gRNA complexity, when compared to a newly isolated strain. The laboratory lifestyle is clearly different from the lifestyle in the wild, with passages through insect and vertebrate hosts and corresponding adaptive changes in energy metabolism.

#### 4.4.7 Editing in Plant Mitochondria

RNA editing in plants is not nearly as dramatic as in trypanosomes, but it is found in both mitochondria and chloroplasts [156]. The most recent and comprehensive review is by Maier and colleagues [157]. A very important distinction is that, in this case, editing does not involve the insertion or deletion of nucleotides as specified by guide RNAs. Rather, most of the editing reactions lead to specific C to U conversions, but a few U to C conversions have also been observed. While the detailed mechanism is not understood, there are indications that, at least in mitochondria, RNA editing may be a simple deamination ( $C \rightarrow U$ ).

It is obvious that until RNA editing in plant plastids was discovered, there was potential confusion in identifying or translating open reading frames. Some apparent differences between the plant mitochondrial genetic code and the universal genetic code disappeared when editing was considered, and sequence differences between homologous plant mitochondrial genes also disappeared after editing was discovered independently in three laboratories in 1989 [158–160]. Most significantly, editing creates the AUG initiation codon in the *nad1* transcript of wheat mitochondria [135] and thus defines the ORF. Similarly, in potato mitochondria editing of the *rps10* transcript creates the correct start codon and a new stop codon. Therefore, editing must be considered in all analyses of newly sequenced plant mtDNAs.

The basic approach to identify editing is to clone cDNA sequences from organelle mRNAs and then compare the sequences with the corresponding sequences on the mt genome. The eight genes and their transcripts for subunits of complex I of wheat mitochondria (*nad1*–7,9) can serve to illustrate the extent of editing observed in mitochondria (see reference 157 for a summary of the results and the primary references). Editing sites appear to be distributed at random in most transcripts, and the density of sites per unit length of RNA is variable, ranging from 5.5 per 1000 nt in *nad5* to 59.3 per 1000 nt in *nad3*. Exon 2 of *nad4* and exons 1, 2, and 3 of *nad5* are not edited at all, whereas the flanking exons are edited. An interpretation of this curious fact is still highly speculative, and it includes the possibility that a portion of the gene represents the incorporation by homologous recombination of a cDNA obtained from reverse transcription of the edited mRNA [157]. There is to date only one plant mitochondrial mRNA that is not edited at all, but it may be a special case, as it is the transcript of a chimeric gene composed of 3' regions of the 26S rRNA gene, an unknown sequence and a part of the 26S rRNA gene. Structural RNA (e.g., rRNA) genes in plant mitochondria are subject to very limited editing. The unedited transcript was discovered because it had been found to be responsible for the Texas male sterility of maize [161].

What makes a particular cytidine a substrate for the presumed deaminase activity? It appears to depend on local sequences. This conclusion was reached from the observed editing in partial gene sequences in chimeric transcripts, which were made from chimeric genes created by the frequent recombination events in plant mt genomes. For example, a 193 nt fragment of exon 1 of *cox1* is edited at exactly the same positions as found in the intact *cox1* mRNA [157].

Editing sites have been found at lower frequencies in rRNAs, tRNAs, in intergenic regions of multicistronic transcripts and in the cis-spliced or trans-spliced group II in-

trons mentioned above. The C to U conversions can be found in stems of secondary structures where a perfect A-U base pair would presumably be stabilizing relative to an A-C base pair. Editing may therefore contribute to the formation of the secondary structure necessary for the splicing or processing mechanism.

It is clear that editing is a posttranscriptional event, since partially edited transcripts can be routinely identified in plant mitochondria. Furthermore, when such partially edited transcripts are analyzed further, it is found that the editing process is random, that is, it does not proceed with any apparent polarity, in contrast to the editing process in trypanosome mRNAs. This observation is consistent with a recognition of the site to be edited based on local sequences by the enzyme catalyzing the deamination, (or other, more complex, reactions such as base or nucleotide exchange). Since at any time a population of partially edited mRNAs exists in mitochondria, one can speculate whether such mRNAs are translated, presumably into peptides with amino acid sequences differing from the normal peptide derived from fully edited mRNA. Mechanisms may exist that prevent partially edited mRNAs from associating with the translational machinery. In one test case the *atp9* subunit was produced from an unedited transcript in the nucleus of transgenic tobacco as well as from the endogenous mitochondrial *atp9* gene (and edited transcript). The nuclear *atp9* transcript also encoded a signal sequence for mitochondrial import, and thus both the normal and the abnormal *atp9* peptide (sequence differences at seven positions) were available for assembly of complex V. The large fraction of plants with cytoplasmic male sterility were interpreted to result from the assembly of inactive ATPsynthases [162].

Efforts to establish *in vitro* systems for RNA editing from mitochondrial extracts are showing promise [157,163], and with the help of such *in vitro* experiments it will be possible to answer one of the more intriguing questions: how are cytidines selected for deamination? So far, an analysis of neighboring regions has not shown any sequence conservation (consensus sequence) nor given indication of localized secondary structures.

#### 4.4.8 Control of mRNA Levels by Turnover

The integration of mitochondrial gene expression with the synthesis and import of peptides encoded by the nuclear genome has long been recognized as an important issue. One need not belabor the point that there must be a coordinate expression of many genes to make a functional electron transport chain, and a unique kind of complexity derives from the need for coordinating the expression from two genomes in separate subcellular compartments. In this chapter the focus is on the mt genome, which can probably be considered to play a subordinate role, since nuclear gene products are required for all essential processes: replication, transcription, and translation. An interesting and important question is whether there is any signal from the mitochondria that directly influences gene expression in the nucleus.

A priori there are several levels at which control could be exerted: 1) transcription of the genome; 2) stability of the mRNA; and 3) translational control, that is, efficiency of initiation or elongation. The consequences of the joint activity of the first



two steps can be measured by the steady-state levels of the mRNA(s) under consideration. It is also quite apparent that the rate of processing of the mitochondrial polycistronic transcripts can be an important factor—especially evident in the case of vertebrate mitochondria.

The structure of the promoters on mt genomes is quite simple, as described in an earlier section, and it is apparent that these promoters are not differentially active in combination with a wide variety of transcriptional activators or repressors. Instead, the presence of the RNA polymerase in combination with one major accessory/transcription factor (mtTFA in mammals) is likely to determine a steady rate of transcription at all promoters. The levels of the mitochondrial RNA polymerase and the mtTFA are most likely controlled by nuclear factors (see Section 4.2.2.3).

It has already been discussed how transcriptional termination in mammalian mitochondria is responsible for the synthesis of unequal amounts of rRNAs relative to many of the mRNAs. Obviously, all mRNAs encoded on a polycistronic transcripts are made in equal amounts, and any observed differences in their steady-state levels must be accounted for by differential turnover. Is there any evidence for differential turnover? Mitochondrial mRNAs have been described to have relatively short half-lives, but the whole subject has not been explored in great detail, either in different tissues of a given organism or in different organisms (however, see reference 164). A series of recent reviews appear to have found an insufficient number of studies on mitochondria, and the subject is discussed together with the corresponding situation in chloroplasts [165–167]. Chloroplast development is beyond the scope of this book, meriting its own detailed treatment. Nevertheless, there may be lessons derived from the studies of chloroplasts. There is control in the context of the development of the whole plant, and there are significant responses to environmental cues, notably light. The only comparable system studied extensively with respect to mitochondria is the adaptation of the yeast *Saccharomyces cerevisiae* to changes in external carbon sources.

The earliest studies on mRNA turnover in HeLa cells were conducted by measuring the kinetics of labeling with [5-<sup>3</sup>H]uridine and the decay of labeled mt mRNAs after blocking transcription with 3'-deoxyadenosine (cordycepin). It was shown that because of turnover of mRNAs, rRNA species were significantly more abundant (~50-fold) than mRNAs [164]. Half-lives in the range of 25–90 minutes were reported. Subsequent studies gave conflicting results, in particular, studies involving the destruction of mtDNA (labeled with 5'-bromouracil and irradiated to stop transcription) [168]. It was suggested that turnover is coupled to transcription or processing or both, and that the stability increased after inhibition of transcription. The true situation is likely even more complicated, since rapid mt mRNA decay in fibroblasts can also be observed when protein synthesis is inhibited, either by a nuclear mutation affecting the translational machinery or by the addition of chloramphenicol [57]. It is noteworthy that in these studies not all mRNAs were affected equally. At this time much remains to be learned in mammalian cells, with the impetus derived in part from the study of human patients with various mitochondrial mutations, including mutations that affect mitochondrial gene expression (see Chapter 7).

In yeast there have been a number of interesting and intriguing observations of altered stability of specific mitochondrial transcripts, which remain to be fully understood, but it appears that these observations also establish a link between mRNA stability and the translation of the particular mRNA. This subject is covered fully in Section 4.5.5; a brief description of the phenomenon is given here.

The yeast mitochondrial cytochrome *b* gene is transcribed as a discistronic precursor RNA requiring extensive processing. Two introns have to be removed to form the COB mRNA itself, and a tRNA<sup>Glu</sup> has to be released from the 5' end to create a 5' UTR for translational initiation. Most relevant for the present discussion is the finding that translation of the COB mRNA requires two translational activators encoded by the nuclear genes *CBS1* and *CBS2*. In *cbs1* mutant strains the transcript is degraded rapidly after the tRNA has been cleaved off, and it has been postulated that the Cbp1 protein stabilizes the COB mRNA by an interaction with its 5' UTR, and further, that it may either negatively regulate a nuclease or induce a processing event which makes the COB mRNA resistant to nucleases. (see Dieckman and Staples [168b]). CBP1 and CBP2 are not the only mRNA-specific translational activators in yeast mitochondria. Translation of the COX3 mRNA requires three proteins (nuclear genes *PET54*, *PET494*, and *PET122*). Similarly, the OLI1 mRNA is dependent on the nuclear gene products *AEP1* and *AEP2*. Translation and stability are affected by the Pet122 or Aep2 proteins, respectively, again interacting with the 5' UTR of these mRNAs.

The primary role of these proteins is most likely to be in translation, but as in the mammalian example described above, mitochondrial mRNAs appear to be protected from degradation when they are being translated.

## 4.5 TRANSLATION OF MITOCHONDRIAL mRNAs

### 4.5.1 Introduction

The mitochondrial translation system is a completely independent molecular machinery for protein synthesis composed of components encoded by the nuclear and mitochondrial genomes. It is not essential in the sense that many cells can grow quite well under conditions where glucose is abundantly available for glycolysis. The main function of the mitochondrial translation machinery is to provide a few peptides for the assembly of the oxidative phosphorylation system. The potential redundancy of mitochondrial protein synthesis was recognized early in the history of yeast genetics. Somewhat later it was discovered, however, that in the absence of mitochondrial protein synthesis the mitochondrial genome is lost for still unexplained reasons [169]. In mammalian fibroblasts mitochondrial protein synthesis can be inhibited more than 95% by a still-undefined nuclear mutation without the loss or even decrease in the amount of mtDNA per cell [57].

A statement encountered frequently in the earlier literature is that mitochondrial ribosomes and general translation factors are similar to their prokaryotic counterparts, but a closer look can identify numerous aspects of translation in mitochondria that differ significantly. Further distinctions have to be made in comparisons of different

organisms. Broadly speaking, the observed differences can be assigned to following major levels, which are discussed in subsequent sections:

1. Changes in tRNA structure and use of altered codons
2. Changes in the fine structure of ribosomes
3. Cis-acting elements (5' and 3'UTRs) of mRNA
4. Initiation factors.

#### 4.5.2 Codon Usage and tRNA Structure

One of the major surprises was discovered when the human and bovine mtDNA sequences were completed and compared. The genetic code was not universal after all! The termination codons, UAA and UAG, defined for both prokaryotes and the eukaryotic cytoplasmic translation machinery did not function as termination codons in mitochondria, and a different set of termination codons was used. Also, additional codons could be used as initiation codons. The data cannot be summarized in simple statements for all species because there are species-specific differences as well. UAA is found as a termination codon in most species examined, but frequently the ORF of a specific mRNA ends with either a U or a UA, and the termination codon is completed by the polyadenylation step. UAG and AGA have been found as termination codons in some species (see Table V in reference 4).

Mammalian mtDNA encodes 22 tRNAs, far fewer than the number available in the cytosol. Therefore, a single tRNA must be able to read all codons of a four-codon family, but it is not yet clear whether in some cases two nucleotide pairs are sufficient, or whether a uridine in the anticodon wobble position can pair with all four third-position nucleotides. A uridine in the wobble position (first position of the anticodon) is frequently modified to pseudouridine in some tRNAs that recognize two-codon families ending in G or A, leading to speculation that this structure prevents misreading the two-codon family ending in C or U. A similar rationale for accurate codon-anticodon recognition has been proposed to explain specific modifications found in the nucleotide immediately following the anticodon [4].

Metazoan mtDNAs encode only one tRNA<sup>f-Met</sup> having the anticodon CAU, and it is the only tRNA for methionine. Not only internal AUG, AUA, and all AUN codons have to be recognized by this tRNA, but the other start codons—UUG, GUG, and GUU—

**TABLE 4.2 Codon Usage in Mitochondria of Various Organisms**

| CODON              | Standard Code | Mammals | <i>Drosophila</i> | <i>Neurospora</i> | Yeasts | Plants |
|--------------------|---------------|---------|-------------------|-------------------|--------|--------|
| UGA                | STOP          | Trp     | Trp               | Trp               | Trp    | STOP   |
| AGA, AGG           | Arg           | STOP    | Ser               | Arg               | Arg    | Arg    |
| AUA                | Ile           | Met     | Met               | Ile               | Met    | Ile    |
| AUU                | Ile           | Met     | Met               | Met               | Met    | Ile    |
| CUU, CUC, CUA, CUG | Leu           | Leu     | Leu               | Leu               | Thr    | Leu    |

must be recognized by this tRNA as well, if it is assumed that all peptides are initiated with formylmethionine. The universal stop codon UGA is translated as tryptophan in mitochondria of many species, and the universal UAA and UAG stop codons are translated as glutamine in some species such as *Acetabularia*, *Tetrahymena*, and *Paramecium*.

It is appropriate to mention here that only a small number of peptides made in mitochondria have been sequenced directly. Many other peptide sequences are deduced from homology with bacterial or other eukaryotic mitochondrial peptides, and codon assignments are often deduced, but not proved, by experiments. In fact, so far it has been impossible to translate mitochondrial mRNAs with a mitochondrial set of ribosomes, tRNAs, and factors in vitro. The earliest attempts failed because the altered codon usage was not recognized, and "mixed" systems were tested, for example, cytoplasmic mRNAs with crude mitochondrial extracts. However, even if the abnormal codon usage is taken into account in such experiments, it has not been possible to translate mt mRNAs in a homologous system. A possible reason for this failure is discussed below.

The complete sequencing of a tRNA was one of the early triumphs of nucleotide sequencing before the modern era of DNA sequencing by the Sanger or Maxam-Gilbert techniques. From this first sequence the familiar cloverleaf secondary structure was proposed, and it has proved to be a universal representation of the structure of all cytoplasmic tRNAs and even chloroplast tRNAs. There are three major loops, referred to as the anticodon loop, the D loop, and the pseudouridylate loop, and the amino acid acceptor stem. When more and more mitochondrial tRNA sequences were elucidated from a large variety of organisms, variations in size and structure of the loops and arms were encountered, although generally most vertebrate and invertebrate mt tRNAs can still be represented by a model resembling the standard tRNA. In extreme cases, either the arm with the D loop or the arm with the pseudo-U loop can be almost completely absent (see reference 4, for examples). In some instances the total length of the presumed tRNA and the secondary structure predicted deviated so much from the norm that additional data had to be interpreted for confirmation. For example, in the nematode *C. elegans* there were 22 tRNA genes, as in other vertebrate and invertebrate mtDNAs, and the anticodon in each of the postulated anticodon arms was compatible with codon usage in these organisms. To show that such unusual tRNA structures are indeed produced, oligonucleotide probes were designed and successfully hybridized to a fraction of small (<150 nt) RNAs from mitochondria of *C. elegans* [4], and the possibility of the creation of "standard" tRNAs by a trans-splicing or RNA editing mechanism was specifically ruled out.

The characteristic 3'CCA end serving as the aminoacyl acceptor site is not encoded in the mt genome of metazoans and other species, and constitutes one of several posttranscriptional modifications. Although this modification might have been expected, many other base modifications encountered upon direct sequencing of tRNAs are more surprising because they require additional enzymes and substrates to be imported into the mitochondria. For example, pseudouridine has already been mentioned; other modified bases include 1-methyladenosine, 1-methylguanosine, N6-isopentenyladenosine, 5-methylcytidine, and N2-methylguanosine [4].

4.5.3 Mitochondrial Ribosomes

When ribosomes in mitochondria were discovered, their “similarity” to bacterial ribosomes became one of the aspects feeding early speculations on the evolutionary origin of mitochondria. However, this similarity was deduced primarily from their sensitivity to chloramphenicol, and insensitivity to cycloheximide, in contrast to eukaryotic cytoplasmic ribosomes. Their hydrodynamic properties were also quite different from those of cytoplasmic ribosomes with a sedimentation coefficient of 55S compared to 80S for cytoplasmic ribosomes, but a comparison with bacterial ribosomes (70S) already gave an indication that the similarity was limited. When the size of the ribosomal RNAs was compared, the discrepancy became even more apparent. A summary of relevant parameters is presented in Table 4.3. The numbers given for the mitochondrial rRNAs are approximate numbers for metazoans, as variations are observed between species from as low as 953 nt for the l-rRNA of *C. elegans* to as high as 1640 nt for the l-rRNA of *X. laevis*; the nematode also has the smallest s-rRNA (697 nt), but the mouse and chicken s-rRNAs contain over 100 more nucleotides than that of the frog (819 nt). There is no equivalent to a 5.8S RNA in mitochondria, and the 5S RNA is found mainly in plant mitochondria, but not in metazoans.

Bacterial and other ribosomal RNAs have been subject to very considerable and fruitful attempts to model secondary structure with the goal of mapping protein/RNA interactions and deriving a structure for the entire ribosome. Sequences from diverse organisms, when compared not as primary sequences but on the basis of secondary structural elements and conserved ribosomal subunit interactions, have revealed sequence blocks that are universally conserved. When mitochondrial rRNAs are included in such comparisons, they fit the same models with some secondary structure elements deleted. Their reduced size arises from deletions of specific internal segments rather than from the deletion of one or few nucleotides throughout their sequence. In other words, the “active sites” in this ribonucleoprotein complex have been highly conserved to catalyze the universal steps in peptide synthesis. For example, the formation of a new peptide bond between the nascent peptidyl-tRNA in the P site and the amino acyl-tRNA in the A site is catalyzed by the “peptidyl transferase,” which is not a conventional enzyme, but rather an activity intrinsic to the large ribosomal subunit. The active site is referred to as the peptidyl transferase center (PTC) and its

TABLE 4.3 Comparison of Ribosomal RNA Sizes in Bacteria, Mammalian Cytosol and Mitochondria

|            | Prokaryotes   | Eukaryotes<br>(mammalian) | Mitochondria             |
|------------|---------------|---------------------------|--------------------------|
| large rRNA | 2900 nt / 23S | 4800 nt / 28S             | ~1600 nt / 16S           |
| small rRNA | 1540 nt / 16S | 1900 nt / 18S             | ~950 nt / 12S            |
| 5.8S       | —             | 160 nt / 5.8S             | —                        |
| 5S         | 120 nt / 5S   | 120 nt / 5S               | 120 nt / 5S <sup>2</sup> |

<sup>2</sup>The 5S RNA is not found in vertebrate mitochondria, but is common in plants.

structure is determined by a combination of domains (IV and V) of the 23S rRNA and several r-proteins, L2, L3, L4, L15, L16, L27. These relationships have been worked out primarily for *E. coli* ribosomes, and the reader is referred to recent comprehensive reviews on this very extensive topic [170–173].

Domain V of the 23S rRNA of *E. coli* contains highly conserved nucleotides, three of which are methylated on the 2' O of ribose. Yeast mitochondrial 21S rRNA is significantly less modified globally compared to *E. coli* 23S rRNA, but the functionally and structurally homologous region has two ribose methylations and one pseudouridine (see reference 174 for references). The conservation of the modified nucleotides is already suggestive of their potential importance in the formation of the PTC, but even more weight can be placed on this interpretation from the finding that the *pet56* mutation in a nuclear gene eliminates a rRNA ribose methyltransferase, that is, an activity responsible for the formation of 2'-O-methylguanosine at G2270 in yeast 21S rRNA. The same activity can methylate in vitro the corresponding G2251 in 23S rRNA of *E. coli* [174]. In other words, failure to methylate the G2270 causes a defect in mitochondrial protein synthesis and a respiration-deficient phenotype. Additional implications of the *pet56* mutation and other mutations in the PET56 gene are discussed by Mason and coworkers [174] who also present another tour de force example of the power of present-day yeast molecular genetics: T7 RNA polymerase was expressed from a nuclear plasmid, but with a signal sequence for import into mitochondria. The *E. coli* 23S rRNA gene with a T7 promoter was transformed into yeast mitochondria. Thus, *E. coli* 23S rRNA made in yeast mitochondria could be examined for the formation of the three expected nucleotide modifications, and at least two have been confirmed so far [174]. This heterologous system obviously has potential for many more interesting comparisons and analyses.

From a determination of buoyant densities in CsCl gradients it can be deduced that mitochondrial ribosomes (1.43 g/cm<sup>3</sup>) have a higher protein : nucleic acid ratio than cytoplasmic ribosomes (1.58 g/cm<sup>3</sup>) [175]. Some of the ribosomal proteins are homologous to their prokaryotic counterparts, but others are unique to mitochondrial ribosomes. A procedure for the large-scale isolation of relatively pure mitochondrial ribosomes from bovine liver has been worked out [175], but an explicit account of all the peptides present is still technically quite challenging. Chances are much better in yeast. It is estimated that there are a total of ~80 ribosomal proteins encoded in the nucleus.

To the extent that homology is a guide, the whole-yeast genome can be scanned for genes encoding ribosomal proteins that are common to bacteria and yeast, but this approach may have its limitations if sequence divergence is extensive. At least 40 proteins are found in the 54S large subunit, of which 30 have been identified from the current Yeast Protein Database [176] as confirmed or likely constituents; 17 of these have clearly recognizable homology or similarity to ribosomal proteins of *E. coli*. A listing of the yeast r-proteins together with their bacterial homologues can be found in a recent review by Mason and colleagues [174]. Unfortunately, a standard nomenclature has not yet been adopted for the yeast r-proteins, making cross-references to bacterial r-proteins somewhat confusing. The identification and cloning of yeast mitochondrial ribosomal protein genes has made it possible to examine the function of

each of these proteins and even domains within each protein by the powerful techniques of molecular genetics. Constructs made in vitro can be substituted for the wild-type gene and the effect of specific mutations on respiratory capacity can be examined in vivo. Guided by the insights derived for the peptidyl transferase center in *E. coli* the precise role of the presumed homologues in yeast mitochondria can be investigated, as exemplified by the work of Mason and colleagues [174]. Many of the detailed conclusions are beyond the scope of this treatise, but a few interesting findings provide provocative contrasts or additional confirmations of structural relationships deduced for one system. Among the examples one can cite the surprising fact that, while L27 is not essential in *E. coli*, the homologous yeast mitochondrial r-protein Rml27p is absolutely essential, and, moreover, conserved as well as nonconserved domains must be present. It was also speculated that the additional mass of the yeast mitochondrial r-protein Rml27p may, in combination with other peptides, provide a "surrogate for the 5S ribonucleoprotein complex" found in prokaryotic and cytoplasmic ribosomes [174].

Other proteins found exclusively in mitochondrial ribosomes have been identified by chance through genetic experiments. For example, suppressor genes of a mutation in a mitochondrial translational activator protein *PET122* have been characterized and identified as nuclear genes for ribosomal proteins that are not found in other prokaryotic or eukaryotic ribosomes [177,178].

A straightforward and definite classification of mitochondrial ribosomal proteins is possible when their genes are actually encoded by the mtDNA. No such genes are found in metazoan mitochondrial genomes, but in yeast there is one such gene (*VAR1*), and in plant mitochondria a variable number is found, depending on the species. Significantly, the *VAR1* gene is not an r-protein gene with homologues in prokaryotic ribosomes which failed to be translocated to the nucleus in the course of evolution. It is rather unique, and as the name implies, it encodes a variant protein, with the most curious variations in different strains of yeast. The observed variation in size is the result of changes in coding sequences at one or more of four different locations: at two of these a 46-base pair cluster of GC can be inserted, and at the two other positions expansions or contractions (in phase) of AAT repeats are observed (codons for Asn). Except for the GC clusters, most of the gene consists of As and Ts. All versions of the protein are active, and, when missing, mitochondrial protein synthesis is defunct, that is, the gene is essential. Since mutations in the *VAR1* gene or nuclear mutations affecting its expression would block mitochondrial protein synthesis and cause a deletion of mtDNA, such mutations are rare. The functional role played by Var1p is still subject to speculation. Proposed functions include an involvement of the peptide in small subunit assembly or a participation in translational initiation requiring message-specific translational activators (see below) and the small ribosomal subunit. In ongoing experimental approaches to study this r-protein the gene has been reengineered for expression from a nuclear plasmid and promoter, and import into mitochondria in which the endogenous gene has been knocked out [174,179]. Because the gene functions perfectly well when expressed from a nuclear location, arguments cannot be supported that its retention in yeast mtDNA has a selective advantage or functional significance.

It has already been mentioned in another context that mt ribosomes, like prokaryotic ribosomes, are sensitive to chloramphenicol but insensitive to cycloheximide. This property makes it possible to study mitochondrial protein synthesis in intact mammalian cells in tissue culture, for example, or to eliminate residual contamination of protein synthesis in isolated mitochondria by the cytoplasmic translation machinery. The binding site for chloramphenicol can be modified by a mutation in the rRNA gene, resulting in chloramphenicol-resistant mammalian cell mutants in tissue culture. Such mutants were the first cytoplasmic mutants of mammalian cells to be isolated [111,180]. Somewhat later, other phenotypes resulting from mutations in mammalian mtDNA were described: oligomycin and antimycin resistance can be due to mutations in peptides of complex V or complex III, respectively [181–183]. The isolation of such mutants also constituted one of the first indications that these peptides were encoded by mtDNA. A further discussion of mitochondrial mutations in mammalian cells is presented in Chapter 7.

#### 4.5.4 *cis*-Acting Elements of Mitochondrial mRNA

The extreme economy of spacing genes on mammalian (and most metazoan) mt genomes has been confirmed by the analysis of the polycistronic transcripts and their processing into rRNAs, tRNAs, and mRNAs. The process yields mRNAs with no 5'UTR, and the terminal stop codon of some being completed by the posttranscriptional polyadenylation reaction. There is also no 5' capping on mitochondrial mRNAs. By contrast, almost all cytoplasmic mRNAs have caps, have 5'UTRs, varying in length from 20 to hundreds of nucleotides, and have 3'UTRs of a bewildering variety and size, sometimes exceeding the entire coding sequence. The importance of the 5'- and 3'UTRs has become recognized only during the last decade when it became quite clear that these structural elements of an mRNA can serve very crucial functions in determining the intracellular localization of the mRNA, the stability (half-life) of the mRNA, or the efficiency with which an mRNA is translated [165,184]. In each case, the primary sequence or the secondary structure resulting from the sequence can act as the *cis*-acting elements that, in conjunction with trans-acting RNA-binding proteins, govern the behavior of the mRNA.

One has to wonder whether similar mechanisms are applicable to mammalian mitochondrial mRNAs, and, if so, the relevant *cis*-acting elements must be contained entirely within the coding sequences. The same dilemma is not encountered for mRNAs in mitochondria of yeast and plants, for example. The genomes are much larger, lengthy intergenic sequences exist, and about 20 transcription start sites have been mapped on the yeast, *S. cerevisiae*, mt genome. Polycistronic transcripts containing only a small number of ORFs are processed to leave significant stretches of A+U-rich 5'UTRs and 3'UTRs in the mature mRNAs. Similarly, plant monocistronic mt mRNAs have long sequences flanking the open reading frame. By contrast, in *S. pombe* the mt mRNAs are also short and lacking 5'- and 3'UTRs.

The importance of the 5'UTR of yeast mt mRNAs has been convincingly demonstrated by genetic experiments. It is the target for imported proteins controlling the turnover and translation of these mRNAs, as will be elaborated below. At this time



only one specific sequence 5'-UUUAUA-3' has been demonstrated to have significance, since it is found in all yeast mitochondrial mRNAs and constitutes part of a binding site for a 40-kDa protein. The binding site appears to be made up of a helical region containing this sequence and an upstream segment of unpaired nucleotides of undefined sequence. This protein is found to interact with all mitochondrial mRNAs of *S. cerevisiae*, and it therefore must be distinguished from the mRNA-specific factors required for stabilization and translation of the individual mRNAs (see below). Other structural characteristics (primary sequence, stem loops) responsible for protein recognition have not yet been further defined. In particular, there is no evidence for the presence of sequences that might be involved in a Shine-Dalgarno type of interaction characteristic of the association of the small ribosomal subunit with mRNAs in prokaryotes.

All the 3' termini of yeast mitochondrial mRNAs have the conserved dodecamer sequence 5'-AAUAAUAUUCUU-3'. It is created directly by endonucleolytic processing of polycistronic transcripts, while the resulting 5' end has to be further processed to yield the mature downstream mRNA. The conserved motif may not be responsible exclusively for specificity of the endonuclease, but may have an additional function in the stabilization and/or translation of the mRNA [133].

#### 4.5.5 Translation Factors

It may be quite astonishing to learn that decades after the discovery of genes, mRNA, and ribosomes in mitochondria there still is no in vitro system for carrying out mitochondrial protein synthesis with components derived exclusively from mitochondria. Only some isolated steps of the entire process have been successfully reproduced in vitro. As a result, it has been difficult to devise an assay for the fractionation and purification of all the required initiation and elongation factors, and our understanding of translational initiation in mitochondria is in its infancy. Two approaches have been partially successful in identification of at least some of the factors. A biochemical approach has been based on assays for factors capable of supporting a limited number of steps of the reaction, and a genetic approach has been successful primarily in the yeast *S. cerevisiae* because of the large number of mutations that have already been selected, screened, and categorized.

Before the mitochondrial factors are described, a brief recall of the factors active in normal cytosolic translation is in order. In prokaryotes there are three factors required for initiation: IF1, IF2, and IF3; in eukaryotes there are five "factors" involved in initiation: eIF1A, eIF2, eIF3, eIF4, and eIF5 [184]. There are two factors needed for elongation, termed Tu and Ts in prokaryotes, and EF1 and EF1 $\beta$  in eukaryotes. Finally there are termination factors (TF). A flurry of activity and the powerful combination of biochemistry and genetics in yeast has led to the elucidation of both the structure and individual functions of these factors in prokaryotes and in the cytosol of eukaryotes. In prokaryotes the three IFs bind to the small ribosomal subunit. F-Met tRNA, the ribosomal subunit and mRNA then form a ternary complex from which IF3 has been released. The complex includes the Shine-Dalgarno sequence and the initiation codon on the mRNA. Finally, the large subunit is assembled, displacing IF1

and IF2 to complete the 70S initiation complex, and elongation is ready to start. In eukaryotes, by contrast, a complex including eIF1A, eIF3, and eIF2, the 40S ribosomal subunit, and f-Met tRNA is assembled, while the mRNA is separately bound by eIF4. The two are combined at the 5' cap site, and a scanning mechanism moves the 40S ribosome to the first AUG, where finally the other factors are released, and the large 60S ribosomal subunit is assembled with the help of eIF5.

The "factors" are somewhat misnamed, because each eIF consists of multiple subunits whose precise function at each step is still being refined. For example, eIF4 consists of several peptides, one for binding to the 5' cap of a eukaryotic mRNA, another performing the function of a helicase, presumably involved in the scanning of the 5'UTR and the removal of any secondary structure (stem loops). Many of these peptides are now recognized as substrates for one or more serine-threonine kinases, which can modulate the activity of the factor and thus control the rate of initiation of translation, either globally or more selectively with mRNAs having the appropriate cis-acting sequences.

In contrasting the prokaryotic and eukaryotic systems, one should note specifically that in prokaryotes the small ribosomal subunit appears to be positioned directly over the start codon with the help of an interaction between the Shine-Dalgarno sequence four to seven nucleotides upstream from the start codon in the mRNA and a complementary nucleotide sequence on the 3' end of the 16S ribosomal RNA. In eukaryotes the initial assembly occurs at the extreme 5' end (cap), followed by a scanning mechanism to find the start codon. Secondary structure can interfere and must be overcome. The stability of such obstructions, and mechanisms to overcome them, can be additional control points for translational control.

Does mitochondrial protein synthesis follow the prokaryotic or the eukaryotic model? There are no caps and no Shine-Dalgarno sequences in mitochondrial mRNAs. Mammalian mitochondrial mRNAs have no 5'UTR, and hence no need for scanning, but certainly a need exists for proper positioning of the ribosomes over the first codon. In other organisms relatively long 5' UTRs exist, with significant secondary structure. What factors do we need? Which of these are common? Which of these are unique to one or a small subset of organism?

During the last few years a single initiation factor (IF-2<sub>mt</sub>) and three elongation factors (EF-Tu<sub>mt</sub>, EF-Ts<sub>mt</sub>, and EF-G<sub>mt</sub>) have been purified and characterized from animal mitochondria by a strictly biochemical approach. Typical starting material is fresh liver from male Angus cattle, and about 30 g of mitochondria are used for making the initial crude extract [185]. The activity of the initiation factor can be assayed by its stimulation of the binding of f-Met-tRNA to mitochondrial ribosomes (or 28S subunits) and poly(A, U, G) as a template. The EF-Tu<sub>mt</sub> activity was shown to be able to replace the corresponding factor from *E. coli* in the synthesis of polyphenylalanine in the presence of bacterial ribosomes and a poly(U) "message." This is probably one of the clearest indications of the relationship of the mitochondrial factor to the prokaryotic factor. In a similar vein, EF-Ts<sub>mt</sub> can stimulate the exchange of guanine nucleotides bound to *E. coli* EF-Tu, and EF-G<sub>mt</sub> can substitute for *E. coli* EF-G in poly(U)-directed polymerization of phenylalanine with bacterial ribosomes.

The problem of elongation in mammalian mitochondria may therefore be close to a solution, to the extent that we understand the same problem in *E. coli*. Initiation remains a problem awaiting further exploration. Following the prokaryotic example one would expect to find at least two more factors if suitable assays could be designed, but the diversity of 5' UTRs found in mitochondria from different organisms should probably raise one's expectations.

There is also the genetic approach. Although a mammalian mutant cell line with a defect in mitochondrial protein synthesis has been described [56,57,186], it is quite apparent that the mammalian cell in tissue culture is not the optimal system for isolating such mutants. In the most elaborate attempt to date, about 50 respiration-deficient mutant cell lines were isolated and grouped into seven complementation groups, one of which had the phenotype of interest here [187]. This is to be contrasted to the very large collection of yeast, *S. cerevisiae*, *pet* mutants, so-called because they make only small (petite) colonies on media with nonfermentable carbon sources such as glycerol, but grow normally on glucose [59]. Because of the many nuclear genes required for making respiration competent and oxidative phosphorylation-competent mitochondria, many different complementation groups have been identified, and many of these mutants are still frozen away in private collections waiting to be further characterized. Quite a few have been examined in detail, and among them a few have been shown to have defects which have shed light on aspects under discussion in this chapter. It is difficult to select yeast mutants specifically defective in mitochondrial protein synthesis (possibly as suppressors of other mutations?), but the ease of isolation of *pet* mutants makes it possible to obtain a large sample of mutants including those of potential interest for any aspect of mitochondrial biogenesis. Some specific strategies for isolating *S. cerevisiae* mutants defective in mitochondrial translation factors have been proposed by Fox [177].

A complete deficiency of a factor essential for mitochondrial protein synthesis leads for unknown reasons to a total loss of mitochondrial DNA. Only leaky mutations could be identified with sufficient activity to maintain the mitochondrial genome, but below the level required to sustain respiration and growth on nonfermentable carbon sources. The difference in growth rate in the presence or absence of glucose could be exploited to isolate the corresponding genes by complementation, and frequently their identity could be deduced by sequence homology with known genes. Among others, nuclear genes encoding homologs of the bacterial initiation factor 2 (IF2), the bacterial elongation factor G (EF-G), a release (termination) factor, and several amino acyl tRNA synthetases have been cloned and characterized [177] by this approach. The yeast mitochondrial elongation factor EF-Tu has been cloned with the help of the corresponding bacterial gene as a probe in a screen of a yeast DNA library.

The reader is referred to the excellent and authoritative essay by Fox [177] for the other potential strategies and the many intricate aspects of yeast genetics which can be exploited and/or must be considered in the design of selection schemes.

The most unexpected initial discovery was that nuclear mutations could prevent the assembly of an active cytochrome oxidase because of the failure to synthesize a single mitochondrially coded subunit. In other words, the deficiency did not affect

transcription of mtDNA, or have a global affect on mitochondrial protein synthesis, but instead it blocked the translation of a specific mitochondrial mRNA. The first such genes to be identified by such means were *PET494* and *PET111*, which are required for the translation of *COX3* and *COX2* mRNAs, respectively [178]. Since then, the need for specific translation factors has been demonstrated for five of the seven membrane proteins encoded by the yeast mtDNA [188,189]. An eighth mitochondrial mRNA in yeast encodes a hydrophilic ribosomal protein. The translation of *COX2*, *COX3*, and *COB* mRNAs has received most of the attention and therefore our understanding of the potential role of these factors is the most advanced. Again, the lack of a faithful in vitro system has slowed the analysis and has made it dependent on the fortuitous discovery of nuclear mutations and rearrangements of the yeast mitochondrial genome. A summary of present insights will serve to make cautious generalizations where possible, but also to emphasize where information and understanding is still lacking. Some of the conclusions derived from the study of translation in yeast mitochondria may also apply to the translation of at least some chloroplast mRNAs, and similarly, studies with chloroplasts may have relevance and serve as guidance in mitochondrial investigations.

The translation of the *COX3* mRNA requires three nuclear genes found so far, *PET494*, *PET54*, and *PET122*. The gene products are normally present at very small and undetectable levels, but overproduction from multi-copy plasmids allows immunological detection and localization of these gene products exclusively in mitochondria. They are all found associated with the membrane, except that ~50% of Pet54p was found soluble in the matrix (where its additional function in splicing an optional intron in *COX1*-pre-mRNA may be required). Membrane extractions with increasingly harsher conditions indicate that Pet494p and Pet122p are strongly membrane bound (integral membrane proteins? Have transmembrane regions been identified in the peptide sequence?), while Pet54p acts as a typical peripheral membrane protein. A variety of indirect experiments strongly suggest the formation of a complex including all three peptides. In a yeast two hybrid system pairwise interactions were detected between Pet54p and Pet122p and between Pet54p and Pet494p, but not between Pet122p and Pet494p, the two integral membrane proteins. One can speculate that their matrix domains bind and are bridged by the Pet54 peptide. An interaction is also strongly indicated by allele-specific *pet45* missense suppression by a missense substitution in *PET122* [178].

*PET111* required for *COX2* mRNA translation is so far the only nuclear gene identified. In overproducers it can also be detected as an integral membrane protein in mitochondria. For *COB* mRNA translation the nuclear gene products Cbs1p and Cbs2p are necessary. Cbs1 is integrated into the mitochondrial inner membrane, while Cbs2 has properties of a peripheral membrane protein. It is not clear whether they interact directly, or only when called into action in the initiation of translation of *COB* mRNA.

Experimentally there are serious challenges to demonstrate the specificity of the factors for the 5'UTR of a single mRNA, and even more so in the identification of target sequences within the 5'UTRs. Standard molecular genetic techniques can make the constructs of interest for testing, but the problem is to get the genes into the mitochondria. Some early studies took advantage of rearranged or deleted mitochondrial

genomes from which chimeric transcripts are made with the 5'UTRs from one gene fused to coding and 3'UTRs of other genes [190]. Such genomes can be maintained as suppressor  $\rho^-$  genomes in the presence of wild type ( $\rho^+$ ) genomes. A full discussion of the genetics of such systems is beyond the scope of the current discussion. It is sufficient to say that many yeast mitochondrial genomes with deletions and rearrangements have been discovered, and strains carrying such genomes can be constructed and maintained.

A new and potentially very powerful approach is to make the constructs to be tested *in vitro*, and then to introduce them into yeast mitochondria by microprojectile bombardment. Such plasmids can stably transform yeast mitochondria lacking endogenous mtDNA [191]. Mating of appropriate yeast strains can combine the plasmid with an otherwise wild type mitochondrial genome, and recombination can lead to the creation of specific changes in the 5'UTR of the COB gene. The more complex aspects of this procedure are also not to be discussed here in detail. An explicit description and the relevant background can be found in a recent paper by Mittelmeier and Dieckmann [192].

A single or a combination of approaches outlined above has confirmed that the translation of a chimeric mRNA required a translational activator which was specified by the 5'UTR, and not by any other downstream elements. Such a conclusion strongly implies that specific *cis*-acting sequences within the 5'UTR must be responsible for recognition and binding. Simple inspection of sequences has not been fruitful. The regions are often but not always quite long, they are A + U-rich, and they have the capacity for secondary structure formation as predicted by computer modeling. Since each transcript is unique and requires its own factor(s), a search for consensus sequences is potentially productive only in comparisons of the 5'UTRs of the same mRNA from several related yeast strains. Attempts to define functional elements require deletions in the 5'UTRs. Those may be found serendipitously, or induced deliberately by a procedure outlined above.

The COX3 5'UTR is 613 nt long. Deletion analysis has indicated that a 150 nt region between -480 and -330 relative to the start codon is required for translational activation [178]. A more systematic analysis of sequences required for translation has been made with the COB mRNA which has a 5'UTR of 954 nucleotides [192]. Two distinct regions were found to be important: the sequence elements between -232 and -4 are required for translation; observations with additional overlapping deletions confine the required regions to two, between -170 and -104, and between -60 and -4. A further refinement suggested that proximal sequence between -33 and -4 is responsible for the selection of the correct start codon. The latter conclusion was based on the interesting result that the deletion of this proximal sequence did not arrest translation of COB mRNA, but caused the appearance of a novel protein that is a truncated form of cytochrome b, possibly by initiation at the first in frame AUG codon at +96. It was still not possible to distinguish clearly between loss of a binding site for Cbs1p or Cbs2p or even ribosomes, or a change in the three-dimensional structure of the RNA necessary for creating a crucial binding site.

By contrast, the COX2 5'UTR is only 54 nucleotides long, the shortest of all the mitochondrial mRNAs in yeast. In principle it would therefore be easier to define a

target for an activator by site-directed mutagenesis, but the task of getting such mutations into mitochondria is not reduced.

One view of translational activators is by analogy with transcriptional activators. A site on the 5' end of mRNA positions them such that they can interact with and stabilize the basic machinery for translation, in this case one or more initiation factors, a ribosomal subunit or the entire ribosome, and probably the f-Met-tRNA. Interestingly, a Pet122 peptide with a truncated C terminus has been shown to be inactive, but the mutation can be suppressed by mutations at three other nuclear loci which on further examination were found to encode peptides of the small ribosomal subunits. These ribosomal peptides are unique to mitochondrial ribosomes and have no homology to ribosomal peptides from other systems [178]. Other partial or complete deletions or point mutations in the *PET122* gene are not suppressed by these mutated ribosomal proteins, indicating that suppression requires protein-protein interactions. Their distinction from initiation factors may seem almost semantic, but their specificity for individual mRNAs is an important distinction. A second fact to be kept in mind is that so far they have been found only in yeast, primarily because of the attention which has been devoted to this organism. It is probably a good guess that other organisms in which mitochondrial mRNAs have substantial 5'UTRs will have similar nuclear encoded factors; the case for such factors in mammalian mitochondria is more difficult to make. There is no 5'UTR and there is effectively no space to position such an activator upstream of the start codon. The alternative would be to position such a protein downstream in a transient mode, i.e. it would assist in the assembly of the translational initiation complex and then be displaced by the process of elongation. Much luck would be required to define such a gene in mammals by a genetic approach.

A second property which makes the yeast translational activators unique is their firm association with the mitochondrial inner membrane. From this perspective another important function immediately comes to mind. The limited number of peptides made in the mitochondrial matrix have from the beginning been recognized to constitute the most hydrophobic, integral membrane proteins of the various complexes of the electron transport chain and the ATPsynthase (complex V). Their insertion into the membrane may be one of the first and crucial steps in the assembly of complexes III-V. It is therefore possible to consider the membrane associated translational activators somewhat analogous to the components of the membrane complex which attracts polysomes to the endoplasmic reticulum for the cotranslational insertion of membrane proteins and secretory proteins into the ER. However, this analogy cannot be pushed too far or made universal. The mechanism of membrane insertion may be different in mammalian (metazoan) mitochondria where the absence of a 5'UTR makes the binding of such factors to the mRNA impossible (see above). However, it is still feasible to have membrane complexes which interact with the ribosome directly without the need for a site on the mRNA. It is also instructive to consider that two of the four peptides of complex II are integral membrane proteins with three postulated transmembrane segments, but they are made in the cytosol and inserted into the inner membrane during import. It is not obligatory to synthesize all integral membrane proteins of the inner membrane in the matrix. Only very recently organisms

have been found where genes for these anchor proteins have been retained on the mitochondrial genome [193–195], and therefore they must be synthesized in the matrix and integrated into the membrane like the other hydrophobic peptides.

As pointed out by Fox [178] the Var1 peptide is a mitochondrial translation product with mostly hydrophilic domains which becomes associated with a small ribosomal subunit. It is argued that since no nuclear mutations have been found affecting the expression of the VAR1 gene, there may be no translational activators for the VAR1 mRNA. On the other hand, genetic arguments can be made that such mutations would not yield viable cells in the genetic screens used so far, and Fox makes the suggestion that the presence of a VAR1 gene on a plasmid in the nucleus, engineered to have an ORF for translation in the cytosol and import into mitochondria, could provide a parental strain with which mutations blocking the expression of the mitochondrial VAR1 gene might be screened for.

Finally, it has been noted that the absolute number of Pet494p molecules is extremely small, between 2 and 60 per cell, which can be compared with the estimate of ~100 copies of the *COX3* gene per diploid cell [178]. Similarly, the abundance of the other translational activators is in the same range. They therefore appear to be the limiting factors for the assembly of complex IV (cytochrome oxidase) in mitochondria. Not surprisingly, the steady-state levels of *PET494* mRNA is subject to regulation depending on the growth conditions, being up-regulated when yeast is grown in media with nonfermentable carbon sources and down-regulated in the presence of glucose [196]. A more detailed discussion of the coordinate regulation of gene expression and of the assembly of the complexes of the mitochondrial electron transport chain has been addressed in Section 4.2.2.

## 4.6 PROTEIN IMPORT INTO MITOCHONDRIA

Mitochondria are distinct subcellular organelles. The existence of an outer and an inner membrane therefore defines two compartments topologically separated from the cytosol and from each other, with the matrix on the inside and the intermembrane space between the two membranes. The identification of specific proteins in the intermembrane space occurred relatively more recently, but from the time of the understanding of the morphology of mitochondria the question naturally arose: how do proteins get into the matrix? This question was soon broadened to include the proteins found to be associated with the inner mitochondrial membrane.

The problem also became more urgent as soon as it was recognized that the coding capacity of the mitochondrial genome was extremely limited, and that therefore the vast majority of the mitochondrial proteins were encoded by the nuclear genome and synthesized in the cytosol. Pioneering and elegant studies initiated in the laboratories of G. Schatz and W. Neupert with yeast, *Saccharomyces cerevisiae*, and many contributions by the disciples trained in these laboratories and others, have provided answers to increasingly complex and detailed questions. Review articles on the subject have appeared almost annually because of the impressive progress made. Many complementary studies were also conducted with the mold *Neurospora crassa*. Whenever

it has been possible to test key features or mechanisms with mammalian mitochondria, the similarities have been striking, and it appears that the principal insights into mitochondrial protein import derived from yeast will be applicable to mitochondria from many if not all organisms. A question not often raised is the question about the evolutionary origin of the import machinery [63]. With some exceptions, the proteins involved are unique and unrelated to other proteins of eukaryotic cells. From the point of view of the endosymbiont theory of the origin of mitochondria it is clear that the development of a protein import system must have preceded the significant transfer of genes from the endosymbiont cell to the nucleus, that is, it constitutes an early event in the evolution of mitochondria. There was no obvious need for protein import in the prokaryotic ancestors of mitochondria, unless scavenging for "loose" proteins constituted a possible source of nutrients. One might therefore expect closely related and homologous proteins in phylogenetically diverse organisms dedicated to this task, and detailed comparisons between such proteins could be expected to contribute further support to the monophyletic origin of mitochondria (see Chapter 2). Unfortunately, many of the genes have not yet been cloned from other organisms, although one can probably expect some rapid progress in plants. Plants present the additional challenge of understanding the biogenesis of chloroplasts, where similar problems with protein import have to be solved. In chloroplasts there is one additional compartment formed by the thylakoid membranes. Comparative studies on these two organelles promise to be revealing.

As always, the development of experimental approaches and model systems was crucial for progress in this field, and two breakthroughs can be singled out. First, G. Schatz and his collaborators succeeded in using isolated mitochondria from yeast in their import studies, with peptides to be imported also made *in vitro*, using the reticulocyte lysate or wheat germ systems. This approach became feasible when it was discovered that at least *in vitro* protein import was post-translational, in contrast to the situation encountered with protein synthesis and translocation into the endoplasmic reticulum. The second methodological advance is becoming a refrain, or, in more colorful language, "*déjà vu* all over again": the combined approach of molecular genetics and cell biology/biochemistry in yeast has facilitated the analysis in unprecedented detail, because of the ingenuity of selective methods and the efficient way in which mutants can be analyzed from gene to protein, its localization and function *in vivo* and *in vitro*.

According to a recent expert review by Schatz [63], there is still some question whether protein import is posttranslational or cotranslational *in vivo*. The absence of a prominent coating of mitochondria by polysomes (as in the rough endoplasmic reticulum) argues against cotranslational import, but it can occur, since ribosomes have been found to be associated with mitochondria and specific cDNAs have been cloned from mRNAs attached to mitochondria [197]. Polysome attachment was specifically promoted in these experiments by slowing down protein synthesis with cycloheximide.

A problem which was solved in broad outline quite early in this field was concerned with the distinction between proteins made in the cytosol destined for import into mitochondria, and those which were unrelated to any mitochondrial function and



had to be discriminated against by the import machinery. All proteins to be imported were found to have a signal sequence (or targeting sequence) at the N-terminal. It consists of 20–30 amino acid residues, and is usually cleaved off upon import on the matrix side of the inner membrane. The functional significance of such a signal sequence was demonstrated most convincingly by showing that almost any protein, for example, the cytoplasmic protein dihydrofolate reductase (DHFR), could be imported into mitochondria when it was equipped with such a signal at the N-terminal [198–200]. Since other sequences within mitochondrial proteins will become relevant in the discussion of targeting to the inner membrane or the intermembrane space, the N-terminal sequence in the present context will be referred to as the “matrix targeting signal.” Many such sequences have been characterized to date in the hope of defining criteria which would allow one to predict simply from inspection whether a protein, known from its cloned and sequenced gene, was a mitochondrial protein [201–203]. The number of such sequences was surprisingly diverse in length and composition. The major distinguishing feature appears to be the ability to form an amphiphilic  $\alpha$ -helix with positive charges on one side when viewed down the long axis [202]. No obvious distinctions are found between yeast and mammalian cells, for example.

The targeting sequence interacts with receptors on the mitochondrial surface, but its removal on the matrix side is appropriately discussed here also, since sequence recognition within or near the matrix targeting signal by one or more highly specific endopeptidases is required. An exhaustive survey of the literature is impossible, but a few examples will illustrate some major discoveries and generalities. Human patients diagnosed with citrullinemia and a defect in ornithine transcarbamylase (an enzyme in the urea cycle) prompted efforts to understand this mitochondrial enzyme, leading Rosenberg and his colleagues to perform some path breaking studies on mitochondrial protein import in mammalian cells. It was discovered that the matrix targeting signal of OTC was processed in two steps [204, 205]. A survey of proteolytic cleavage sites identified amino acid motifs recognized by the matrix proteases [203]. In general, such studies have been helpful in identifying cleavage sites in the maturation of mitochondrial proteins (the “Arg2 rule”), but an absolutely certain prediction is still not possible in all cases.

Finally, the cDNA for the  $\beta$  subunit of the mitochondrial processing peptidase was cloned from rat liver, and sequence comparisons established relationships with a proposed family of metallopeptidases [206]. A few years ago several of the prominent contributors to this field have established a uniform nomenclature for the mitochondrial peptidases responsible for protein maturation after these laboratories had independently purified proteins, and characterized genes from rat liver, *Saccharomyces cerevisiae*, *Neurospora crassa*, and potato [207]. Three major activities are recognized at this time:  $\alpha$ -MPP and  $\beta$ -MPP are the subunits of the mitochondrial processing peptidase responsible for the initial cleavage of the targeting sequence. MIP (mitochondrial intermediate peptidase) carries out the second cleavage required for maturation of some matrix proteins, removing an additional eight amino acids from the N-terminal. For proteins sorted into the intermembrane space (see below) sorting signals have to be removed as well, and at least two integral membrane proteins with peptidase activity have been identified. They are designated IMP I, and so on, and it appears that

they are heterodimers. An alternate approach to cloning the corresponding peptidases from yeast (originally designated MAS1 and MAS2 [208]) will be discussed below.

The import of a protein into the matrix requires the traverse of two membranes and hence the existence of a mechanism which allows a large, water-soluble polypeptide to pass through the lipid bilayers. Before discussing the proteins involved, and how they were identified and assigned their specific roles, it should be stated that this discussion will use the new nomenclature adopted by the leaders in the field [209]. It therefore becomes possible to talk about the "Tom and Tim machines" [64] and remove at least one potential type of confusion. Tom proteins are integral (or peripheral?) membrane proteins of the outer membrane participating in protein import. Tim proteins are integral membrane proteins located in the inner membrane and participating in the same process. Additional soluble proteins with distinct names are carrying out important functions on the cytosolic side of the outer membrane, and similarly, a variety of soluble proteins on the matrix side have equally essential roles in the overall process. Tom and Tim proteins are distinguished further by numbers corresponding to their molecular mass in kDa. For example, Tom20, Tom22, Tom37, Tom70, and so on represent outer membrane proteins of the import machine.

It is difficult to do justice to the elegance and ingenious design of the many experiments which have made the system less and less of a black box, or a few rectangles symbolizing a pore through the membranes. But before losing ourselves in details, an outline and some highlights will provide perspective and allow the formulation of guiding questions.

A globular, soluble, cytoplasmic protein, when provided with a matrix targeting signal at the N-terminal was found to be imported into yeast mitochondria *in vitro* and *in vivo*. Therefore, the targeting signal had to be recognized by a receptor on the mitochondrial outer surface. Simple notions of polypeptide tails "diving" into lipid bilayers were entertained only temporarily. Since the process was post-translational, one had to assume that the protein was in a folded form, and in fact the test protein, DHFR, was shown to be active before import, and after import. A simple but clever experiment provided evidence, however, that the protein had to be unfolded to be imported: When the tertiary structure of the protein was stabilized by the binding of a ligand (methotrexate), import was prevented. From this and related experiments the notion was generated that mitochondrial proteins are denatured in the process of import and threaded through an import pore as extended polypeptides. This reduces the required pore size, but creates a problem at the beginning and at the end: the protein has to be unfolded first, and the protein has to be refolded once it has arrived on the inside. More will be said about this later.

Since two membranes are involved, one could *a priori* imagine the import process to occur in two stages, with the intermembrane space being an intermediate, temporary location. However, matrix proteins were never found there, even transiently, and no conditions were found which would have arrested the process at such an intermediate stage. It could be shown that proteins had their targeting signal removed before they were even completely transferred to the matrix. For example, at lower temperatures *in vitro* translocation can be initiated, but the peptide remains "stuck" in the membrane. The interpretation was that the N-terminal protruding into the matrix was

subject to proteolytic processing by the matrix peptidases while the remainder of the peptide was still in transit. Another observation made with the electron microscope was that the inner and outer membrane were frequently in close contact, and it was hypothesized that these were locations where a complex of proteins in the outer and inner membrane created a channel traversing both membranes for protein import. Such a structure may indeed exist, but only transiently (see below).

A key observation made relatively early in the course of these investigations was that the isolated mitochondria used in the *in vitro* experiments had to be in a state where a membrane potential was maintained. The addition of ionophores or uncouplers destroyed the membrane potential and also made these mitochondria incapable of importing a peptide. This was and continues to be a puzzling observation, although attempts to rationalize it have been made. In general, current thinking attributes to the membrane potential a driving force which pulls the positively charged matrix targeting signal across the inner membrane, since the inside is negative relative to the outside [210]. This problem is part of a larger question about the energetics of the process. What are the thermodynamic parameters which make this process feasible, efficient, and essentially unidirectional. Electrophoresis of the targeting signal across the inner membrane is part of but not the complete answer.

It now remains to understand each of these processes in more detail and to explain how the molecular architecture of the Tom and Tim machines is designed to execute translocation of a polypeptide across two membranes. Today it is clear that each of these "machines" consists of a large number of different peptides which were not discovered all at once. A genetic approach in yeast proved invaluable. In pioneering studies Yaffe and Schatz [62] were the first to isolate and characterize yeast mutants defective in general protein import. Recognizing that mutations affecting all mitochondrial functions might be lethal, a collection of temperature sensitive mutants was screened for the inability to import the  $\beta$  subunit of the F1-ATPase at the nonpermissive temperature, 37°C. The assay was based on a clear distinction in electrophoretic mobility between the precursor in the cytosol and the mature (processed) peptide after import. More than a thousand mutagenized strains were screened, and in the first publication two nonallelic, recessive *mas* mutants (*mas1* and *mas2*, mitochondrial assembly) were characterized in more detail. As described expertly and in some detail by Yaffe [211], such mutants can be analyzed for import and mitochondrial function *in vivo*, and isolated mitochondria can be further investigated *in vitro*. Specificity, observed processing, dependence on a membrane potential, and protection of the imported protein against exogenous proteases were among the criteria defined for import experiments.

Over the years many more *mas* mutants were characterized and the corresponding genes cloned. As described above, they were renamed Toms and Tims, but can be found in the original literature under their original "mas" designations. Not all Tom and Tim peptides were discovered from mutant screens and cloning. A number of peptides, especially in the outer membrane, were first defined by crosslinking studies to already known components.

However, a logical start is with the accessory proteins in the cytosol. It was first discovered by Schekman's laboratory [212] that a subset of stress proteins was re-

quired for the translocation of secretory proteins across the endoplasmic reticulum membrane and mitochondrial proteins into the matrix. It is now recognized that proteins such as those belonging to the hsp70 heat-shock protein family bind to hydrophobic domains of denatured proteins, thus stabilizing an unfolded state, or they may bind already to the nascent peptide emerging from the ribosome during synthesis. Matrix proteins are also bound by these "chaperones," so called because they are believed to chaperone a peptide to its eventual destination, or to assist it in folding into its functional tertiary structure. A matrix protein is kept in an unfolded state by association with cytoplasmic hsp70 proteins and is able to present the matrix targeting sequence to the receptor on the outer mitochondrial surface. When import has been initiated, the chaperone proteins are postulated to be sequentially released as more and more of the peptide is transferred into the mitochondrion. The dissociation of the chaperone from the imported peptide requires ATP hydrolysis. While hsp70 family members are participants in protein translocation into several subcellular organelles, a factor which is exclusively engaged in mitochondrial import has also been discovered. It is also an ATP-dependent chaperone consisting of two subunits (30 and 32 kDa, respectively) and is referred to as MSF (mitochondrial import stimulating factor). The two chaperones, hsp70 and MSP, appear to be involved in distinct import pathways which differ in the nature of the receptor on the outer surface and in the final destination of the imported protein (see below). Their behavior has been contrasted in a review by Mihara and Omura [213].

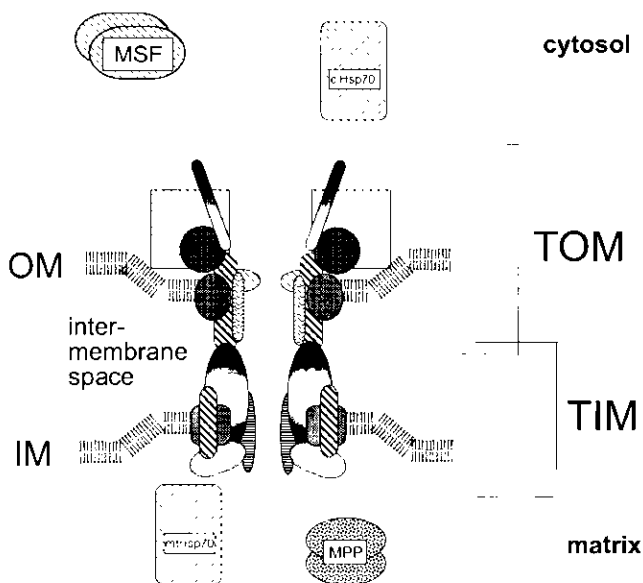
On the inside (matrix) of the import channel the unfolded peptide is received by a closely related matrix hsp70 chaperone whose function is to prevent misfolding or premature folding, or the formation of aggregates. Again, ATP hydrolysis is required for its eventual removal. However, the peptide is not released directly into the matrix, but is delivered to a large complex made of hsp60 and cpn10 peptides and referred to as chaperonin. These peptides are related to the bacterial chaperones GroEL and GroES. Further ATP hydrolysis leads to its eventual release into the matrix as a mature, biologically functional soluble enzyme. This is the simplest scenario. Increasingly more complex situations arise when the protein after import into the matrix becomes associated with other heterologous subunits and integral membrane protein domains on the inner membrane. A relatively simple example is the assembly of the flavoprotein and the iron-sulfur subunit of succinate dehydrogenase with each other and with the two membrane anchor proteins to form complex II, and a more complex problem is the assembling of peptides of the  $F_1$  oligomeric complex ( $\alpha_3\beta_3\gamma\delta\epsilon$ ) and the  $F_0$  oligomeric complex of the ATP synthase (complex V; see Chapter 5.5). Relatively little is understood yet about the pathway of assembly of these complexes (see Chapter 5). For example, the absence of the membrane anchor in complex II prevents the assembly of the peripheral membrane complex of succinate dehydrogenase [214]. It is therefore not surprising that the hsp60/cpn10 chaperonin complex is not the only or obligatory activity assisting with protein folding. A complex has been identified in the matrix made of mhsp70, Mdj1 (a mitochondrial DnaJ homologue), and mGRpE. A mitochondrial proline isomerase (belonging to the cyclophilin family) has been shown to play a role by several laboratories [215,216].

At least two other pathways for peptide translocation “into” mitochondria must be distinguished, in addition to the insertion into the outer membrane. Some peptides are delivered to the intermembrane space. Examples are cytochrome *c* and cytochrome *b*<sub>2</sub>. Others, such as the integral membrane proteins C<sub>11-3</sub> and C<sub>11-4</sub> of complex II, are inserted into the inner membrane. While similar considerations to the above apply to their initial presentation to the mitochondrial import machinery, their interaction with mitochondrial chaperones is less certain, depending on the precise pathway postulated for their topogenesis. Their fate is determined by intrinsic, peptide signals and interactions with other components in the membrane, and may not require the Tim protein complex (see below).

By one of the more recent counts there are at least nine different peptides in Tom and five different peptides in Tim [63,64]. Multiple subunits of one kind may be found in the complexes being formed. One of the major and uncontroversial conclusions about these complexes is that they are not stable over time, but instead are transiently assembled from the interaction of subcomplexes. An attractive and rational hypothesis is that the assembly of one may promote or stabilize the assembly of the other in the juxtaposed portion of the membrane, leading to the temporary alignment of the two channels through the outer and inner membranes (Figure 4.10).

A functional differentiation of the peptides in the Tom complex has been possible on the basis of genetic as well as biochemical studies. Tom40 (the number indicates the molecular mass in kDa) is thought to be the main component responsible for the channel (or pore) through the outer membrane which also includes Tom38, Tom7, Tom6, and Tom5. The latter four proteins are not absolutely required and are therefore believed to regulate or stabilize the pore complex. It is dynamically associated with Tom70, Tom37, Tom22, and Tom20, but mild solubilization procedures can succeed in preserving the Tom70–Tom37 heterodimer and a Tom22–Tom20 heterodimer. Crosslinking studies also support this view, and the interaction can be explained as an interaction between 34-residue-long “tetraco-peptide” motifs present on all but Tom22. Each of these subcomplexes has been assigned a role as receptor, operating by slightly differing mechanisms and with subsets of precursor proteins to be imported. The distinction between these two mechanisms has been elucidated by *in vitro* experiments, but as emphasized by Schatz [63], these receptors may have partly overlapping functions *in vivo*, since only Tom22 is essential for protein import, while Tom20, Tom37, and Tom70 can be deleted by mutation without a very strong effect on mitochondrial biogenesis.

Current speculation [63,64] has the typical positively charged matrix targeting sequence at the N-terminal of a precursor interacting with “acid bristles” (negatively charged segments) on the cytosolic domains of Tom22 and Tom20. The remainder of the precursor may be complexed with hsp70 chaperones. Curiously, and in contrast to the usual involvement of hsp70, no ATP hydrolysis is required here to release the precursor into the channel. The second receptor subcomplex recognizes precursors which are associated with MSF. The MSF delivers the precursor to the Tom70–Tom37 receptor and is then released. In the bound form MSF is an ATPase. Tom70–Tom37 may not be able to deliver the precursor (or the matrix targeting sequence) directly to the pore/channel, but instead passes it on to the Tom22–Tom20 receptor complex, where



**Figure 4.10** Highly schematized representation of the mitochondrial protein translocation machinery. In this view, the complex in the outer membrane (TOM) and the complex in the inner membrane (TIM) are aligned to form a continuous channel through both lipid bilayers. This association may be transient, as explained in the text. Not all peptides are shown. Soluble factors on the cytosol side (MSF and cHsp70) and on the matrix side (MPP and mtHsp70) are also shown. Both TOM and TIM are made up of integral membrane proteins and peripheral membrane proteins. See text for detailed discussion. (After Pfanner and Meijer) (From Reference [64b])

the two pathways appear to join, consistent with the overlapping functions indicated by the genetic experiments.

The role played by the modulator proteins Tom6 and Tom7 deserves some further discussion. Experiments by Alconada and coworkers [217] suggest that Tom6 promotes the assembly of the two receptor subcomplexes with the import pore, Tom40, but in a qualitatively differential fashion. Thus, the absence of Tom6 causes a delay of the transfer of the precursor from the receptor to the channel/pore. Tom7, on the other hand promotes the disassembly of the Tom22–Tom20 subcomplex from Tom40; this disassembly is not necessarily required for normal import, but may be a rate-limiting step in the release of outer membrane proteins such as porin by a lateral release mech-

anism similar to that operating in the release of integral membrane proteins into the lipid phase of the endoplasmic reticulum [64].

The five subunits of the Tim complex (Tim44, Tim23, Tim22, Tim17, and Tim11), have been analyzed extensively by genetic and molecular genetic studies, as well as by crosslinking studies to establish proximities and specific interactions not only among these five peptides, but also between one or more of these peptides with the imported protein (trapped in transit) [218], and with the matrix chaperone (mtHsp70). A mutational analysis has revealed that Tim44, Tim23 and Tim17 are essential for mitochondrial biogenesis and cell viability. The amino acid sequence (hydropathy plot) for Tim17 indicates four membrane spanning segments consistent with its intimate role in channel formation. A similar analysis for Tim23 shows one transmembrane region, and a hydrophilic domain which has been localized in the intermembrane space. Its transmembrane segment is also thought to be part of the channel, while the intermembrane space domain may function as a quasi receptor for targeting sequences emerging from the pore in the Tom complex. Tim11 is similarly divided into a hydrophobic and a hydrophilic domain extending into the intermembrane space, but its function is still less clear.

The largest, Tim44, does not have membrane spanning regions, but is associated as a peripheral membrane protein with the matrix side of the inner membrane via a carboxy-terminal anchor. The most telling subcomplex identified with Tim44 includes mtHsp70 and another chaperone Mge1. The role of "translocation motor" has therefore been assigned to the Tim44-mtHsp70 complex, although competing models still distinguish between an active role (motor) and a more passive role (ratchet). In the first, a conformational stroke driven by ATP hydrolysis actually pulls the protein into the matrix. The model provides a mechanism for unfolding a protein on one side of the membrane(s) as it is being pulled through the narrow channel by the motor. The analogy with motors operating on cytoskeletal elements (microfilaments) has been proposed, but in that case polymeric filamentous structures are involved with regularly spaced binding sites, while in the case of mitochondrial import a single peptide chain with almost any sequence can be imported. Can a chaperone creep along a peptide chain? In the second model a protein in transit is considered to diffuse back and forth (oscillate) with chaperones on the outside and on the inside competing for segments to bind to in a dynamic equilibrium. If the affinity of the chaperones on the inside is slightly higher than that of the outside chaperones, the diffusion through the channel will be biased [219].

The problem of the translocation of a peptide across two mitochondrial membranes can be usefully decomposed into two related problems: 1) what makes it feasible, and 2) what drives the process so efficiently in one direction? A simple pore of appropriate size makes it feasible for a molecule to cross a barrier, and concentration gradients will determine the direction. Mitochondrial protein import has long been recognized to be more akin to active transport, first because of the recognized need for a membrane potential, and later because of the involvement of the chaperones and their associated ATPase activities. In active transport (ion pumps) stoichiometric amounts of ATP are hydrolyzed per mole of ions transported, but the total ATP con-

sumption per peptide imported is not only not known, but also quite variable depending on the protein. Requirements range from no ATP needed at all, to ATP needed only on the outside, ATP needed only on the inside, and ATP needed on both sides. The conditions for manipulating external and/or internal ATP concentrations in vitro in order to study import requirements have been described [220], and some general conclusions can be stated from such experiments. An obvious one is that no internal ATP is required if a protein does not cross the inner membrane. If matrix ATP is required, then the peptide or a segment of it will have crossed the inner membrane on its path to its final destination.

By one definition, protein import is defined as the process which leads to the protection of the imported peptide against an exogenously added protease. A simple test of this type will not distinguish between import into the matrix, insertion into the inner membrane, or import into the intermembrane space. Insertion into the outer membrane may also leave a significant domain of a protein susceptible to proteolytic attack from the outside. A further distinction can be achieved by a variety of schemes. Dilution of mitochondria in hypotonic medium causes swelling and rupturing of the outer membrane, leading to the formation of mitoplasts. Outer membrane vesicles can be made and purified for functional analysis and biochemical/immunochemical assays of composition. Well characterized antibodies, especially monoclonals, directed against specific epitopes can also give useful information about the location and orientation of a protein of interest. Many experimental details on specific experimental protocols can be found in the expert review by Yaffe [211], and in a large collection of reviews of methods in Volume 260 of *Methods of Enzymology* (1995).

Additional considerations are required for proteins imported into the intermembrane space, and for proteins inserted into the inner membrane with more than one transmembrane segment. Sorting into the intermembrane space has been proposed to occur by one of two models developed with cytochrome  $b_2$  as the model protein. Both make use of a matrix targeting sequence at the N-terminal followed almost immediately by another short peptide sequence for targeting. Neither of them can be found on the mature protein. The models differ in their view of the precise role of the second targeting sequence. In the first, the peptide is translocated into the matrix following the path of a normal matrix peptide. Removal of the matrix targeting signal exposes the second targeting sequence whose function is to initiate a second translocation of the peptide in the opposite direction from the matrix into the intermembrane space. Presumably a receptor and pore exist for this purpose. In the alternative model the second peptide signal serves as a stop-transfer signal similar to such signals found in protein translocation across the endoplasmic reticulum. In this case, the matrix targeting signal initiates translocation and reaches the matrix for processing, but the imported peptide is trapped by the second signal inside the pore of the Tim machinery, while further translocation across the Tom channel takes place until the entire peptide is found in the intermembrane space. A proteolytic cleavage close to the outer surface of the inner membrane would liberate the protein as a soluble constituent in the intermembrane space. Integral membrane proteins with peptidase activity have been identified (see above) which could perform this cleavage.



Several inner membrane proteins have multiple transmembrane segments. As examples one can consider the two membrane anchor proteins of complex II, which interact with the succinate dehydrogenase heterodimer composed of the iron-sulfur subunit and the flavoprotein. The anchor proteins ( $C_{11-3}$ ,  $C_{11-4}$ ) are synthesized in the cytosol in most organisms, although a few exceptions have been found where one or more of these peptides are encoded by mtDNA and made in the matrix [193–195]. The peptides are relatively small (15, 12–13 kDa, respectively), and hydropathy plots have suggested three transmembrane segments, with the relatively large N-terminal domain exposed to the matrix. The problem of inserting these peptides into the inner membrane is conceptually very similar to the problem of inserting membrane proteins with multiple transmembrane regions into the ER and plasma membranes. Favored mechanisms include a combination of N-terminal and internal membrane targeting signals, and stop transfer signals, and hence an insertion of the peptide into the membrane in a sequential, loop by loop fashion. One aspect of such models is that a loop with two peptide chains has to be accommodated in the import channel. A radically different mechanism is suggested by the existence of organisms in which the anchor protein is made in the matrix. Here the peptide clearly has to be inserted into the membrane from the inside. Thus, one should consider complete import into the matrix from the cytosol followed by insertion into the membrane even in the much more common situation of nuclear-encoded anchor proteins. There is an additional potential complication which will be fully considered in a later chapter. It is accepted that the two anchor proteins interact with each other and also quite intimately with the iron-sulfur subunit on the matrix side. Experiments have shown that a mutation of the  $C_{11-3}$  anchor peptide causes a failure of the assembly of the SDH complex as well [214]. Therefore, it remains to be seen whether in the case of the electron transport chain complexes subunit interactions play a significant role in the final localization and orientation of the hydrophobic IMPs.

The field continues to expand. In one of the latest reviews Pfanner addresses the import of polytopic inner membrane proteins which include the ADP/ATP carrier, the phosphate carrier, and other carriers with three transmembrane domains per peptide, the functional protein being a dimer [221]. It is concluded that after sharing some of the Tom machinery, the pathway of import into the inner membrane diverges quite dramatically from the pathway for matrix proteins. In this view, some Tim proteins (Tim 23, Tim 17) form a channel for import into the matrix, while others (Tim 22, Tim 54, Tim 12) form a distinct complex for receiving the polytopic membrane protein prior to its release as a “free” integral membrane protein that can subsequently dimerize.

The discussion so far has rested heavily on experiments conducted with *Saccharomyces cerevisiae* and *Neurospora crassa* mutants and mitochondria. Will there be dramatic differences or novel concepts to be discovered in other organisms? Some experience with mammalian mitochondria and with plant mitochondria would lead to the conclusion that the basic mechanisms have been conserved. Tissue specific and developmentally regulated differences in efficiency may be found, but the use of matrix targeting sequences and the proteolytic processing are universal aspect of protein import, and the Tom and Tim machinery may show differences only in detail.

## 4.7 IMPORT OF tRNA INTO MITOCHONDRIA

Until now it has been tacitly assumed that proteins are the only macromolecule imported into mitochondria. A highly charged polyelectrolyte such as an RNA would be expected to have difficulty crossing two membranes, and for some time RNA import into mitochondria was considered irrelevant. An exception, the import of the RNA associated with the mitochondrial RNA processing RNase, has already been mentioned in an earlier section (Section 4.3). From sequencing the mitochondrial genomes of diverse organisms it is now becoming apparent that many such genomes do not encode all of the tRNAs required for translation of mitochondrial mRNAs, and thus tRNAs have to be imported. In one of the most extreme cases, mitochondrial tRNAs of *L. tarentolae* [222] and of *Trypanosoma brucei* [223] are all synthesized in the nucleus and imported. This was established first by showing that the purified mitochondrial tRNAs from *L. tarentolae* hybridized exclusively with nuclear DNA, but not with minicircle or maxicircle DNA. A large fraction, but not all mitochondrial tRNAs in plants are imported, and there are significant differences among different species, and even between relatively closely related plants. It has therefore been suggested that the ability to import specific tRNAs has been acquired at different times during the evolution of higher plants [224]. However, it has to be kept in mind that a complete set of such tRNAs is required at all times for translation, and therefore one must imagine that import of a nuclear-encoded tRNA has to precede the loss of the isoaccepting tRNA transcribed from the mitochondrial genome. Illustrating this idea, in *Saccharomyces cerevisiae* there are three lysine isoacceptors: one is made and found exclusively in mitochondria, a second is found exclusively in the cytosol, and a third is found in the cytosol (95%) and in mitochondria (5%) [225,226].

The specific mechanism operating in the import of tRNAs is starting to come into view [223,224,226–228]. Plant tRNA genes have been identified and used to construct transgenic plants in order to demonstrate that a heterologous tRNA is also imported into the mitochondria of the transgenic plant, i.e. to demonstrate that some universal signal/sequence must be recognized [227,229]. An emerging consensus is that the tRNA is associated with the precursor of the cognate mitochondrial tRNA synthetase, and it may be imported in its aminoacylated form, but this conclusion may not apply to all organisms. In *L. tarentolae* mutated tRNA<sup>Gln</sup> that cannot be charged by the synthetase can still be imported into mitochondria as long as an import signal in the D loop is intact [230]. Sequence and structural changes in the imported yeast tRNA<sup>Lys</sup> have been investigated by in vivo and in vitro experiments to show that binding to the synthetase, and the capacity to be aminoacylated are well correlated with the import efficiency [225]. The protein translocation apparatus (Tom and Tim machine) is clearly involved [228], but this raises a problem if the RNA/protein complex is to remain intact. Peptides are thought to be translocated in an unfolded form. Since the known mechanism for aminoacylation of tRNAs does not include any covalently joined intermediate between tRNA and the enzyme, it is likely that the enzyme and the tRNA would have to preserve their secondary and tertiary structure during import to remain non-covalently bound to each other. In support of this idea Entelis and colleagues [225] have demonstrated that when tRNA is nicked, the 3' end can direct mitochondrial import of the 5' end.

#### 4.8 REGULATED PROTEIN DEGRADATION IN MITOCHONDRIA

When yeast and mammalian mitochondria were analyzed biochemically, proteases represented a prominent enzymatic activity. Further examination revealed that proteolysis was greatly stimulated by ATP and in fact was associated with ATP hydrolysis. This protease activity was distinct from the very limited activity required for the removal of the signal sequence from imported proteins, as described above. However, the target of these proteases and the real biological significance of their presence has become clearer only in recent years.

Fractionation of mammalian mitochondrial matrix proteins yielded an ATP-dependent (vanadate-sensitive) protease with properties resembling those of the *E. coli* Lon protease [231]. Cloning of the corresponding genes in yeast and humans has been accomplished within the past few years, and sequence analysis confirmed a relatedness to the *E. coli* enzyme, although the eukaryotic peptides appeared to be longer with sequence extensions having an as yet unknown function. The gene was designated *PIM1* (proteolysis in mitochondria). A striking aspect of these serine proteases is their high molecular mass, due to the formation of an oligomeric (hexamer) functional form.

With the gene identified, the yeast system offered powerful means of establishing the function of the PIM1 protease, since the gene could be knocked out and the physiological consequences could be examined. It may have come as a mild surprise that *pim1* mutants are respiration-deficient and affected in mitochondrial biogenesis [232]. More puzzling is the observation that *pim1* mutants accumulate deletions in mitochondrial DNA. It is not clear whether this is a direct effect, for example overproduction of a Pim1p-sensitive protein with a destabilizing effect on mtDNA. Alternatively, mtDNA stability in yeast is dependent on respiration, and its integrity is compromised in other respiration-deficient mutants. The *pim1* phenotype could be rescued by the expression of the *E. coli* Lon protein in yeast, provided it was expressed with a mitochondrial targeting sequence [233].

What is the target of the PIM1 protease? First of all, it appears to degrade specific peptides such as the  $\beta$ -subunit of the mitochondrial processing peptidase and the  $\beta$ -subunit of the F1-ATPase when cytoplasmic protein synthesis is inhibited. One can consider this an example of regulated protein degradation, and it may be part of the overall response of a yeast cell to amino acid starvation. Furthermore, mutated, misfolded polypeptides imported into the matrix are prevented from accumulating and aggregating by the activity of Pim1p. In the latter case, misfolded proteins have to be "presented" to the PIM1 protease by the mitochondrial Hsp70 system, in conjunction with the activity of two other components, Mdj1p and Mge1p [234]. In the authoritative review by Neupert and coworkers [235] another interesting question is now raised: the mt-Hsp70 system is intimately involved in the normal import of proteins from the cytoplasm, aiding in their folding to their native conformation after translocation through the Tom and Tim machine (see Chapter 4.6). This process also requires ATP. The multiple roles played by the mt-Hsp70 system therefore require that a distinction be made between normal proteins transiently associated with Hsp70 and abnormal proteins to be degraded by the PIM1 protease. Neupert makes the suggestion that a normal protein would be released to the mtHsp60 system for its final folding,

while an abnormal protein may also be released, but failing to fold properly, it may be recaptured by the mtHsp70, and after prolonged dynamic association with the mtHsp70 system the PIM1 protease may finally gain access.

Taking a cue from the existence of a second class of ATP-dependent proteases in bacteria, (referred to as Clp proteases), a search for a eukaryotic equivalent function has been successful in humans [236], but a *Saccharomyces* homologue could not be identified from sequence comparisons with the entire yeast genome [235].

When cytoplasmic protein synthesis is stopped, and proteins made inside the mitochondria now fail to be assembled into complexes in the inner membrane, they are also rapidly degraded. A further examination of this process has revealed the existence of two additional ATP-dependent metallopeptidases responsible for the degradation of nonassembled mitochondrial translation products. These proteases are integral membrane proteins in the inner membrane. From amino acid sequence data the proteins can be assigned to the AAA family of proteins (ATPases associated with a variety of cellular activities) characterized by an ATPase domain comprised of 230 conserved amino acids, and as proteases they represent a distinct subgroup of this family. Another conserved motif is the HEXXH sequence shown to be responsible for divalent metal ion binding. The two peptidases have been named m-AAA-protease and i-AAA-protease, since it has been shown that the active site of one of them is on the matrix side of the inner membrane (m-AAA-protease), while the other has its active site in the intermembrane space (i-AAA-protease).

The m-AAA-protease is a 850 kDa complex made up of heterologous but closely related peptides encoded by the YTA10 and YTA12 genes [237]. Each peptide has two transmembrane helices; a short N-terminal domain and a large C-terminal domain are exposed to the matrix and ATP and metal ion binding sites can be found in these domains. The Yta10p and Yta12p constituents of the complex may have different substrate specificities. A knockout of either gene leads to a failure of cells to grow on nonfermentable carbon sources, that is, the m-AAA proteases on the inner membrane appears to be essential for the assembly or maintenance of the electron transport chain and the capacity for oxidative phosphorylation. In fact, a chaperone-like activity of the m-AAA protease for the assembly of the  $F_0$  complex of the ATP synthase has been suggested based on genetic studies. The proteolytic and chaperone-like activities appear to be independent of each other [235].

The i-AAA-protease is a similarly large complex (850 kDa), but it is made up of a single type of subunits encoded by the *YME1* gene, alias *YTA11*, alias *OSD1*. Multiple independent approaches had identified this gene [e.g., 238], the first time from an analyses of mutants with an increased rate of escape of mtDNA to the nucleus [239]. The Yme1p has been given a broad role in the maintenance of mitochondrial morphology under various conditions, since its absence causes not only a respiratory deficiency at elevated temperatures, but also morphologically abnormal mitochondria. Destruction of the proteolytic activity by a specific point mutation causes a similar phenotype, and this has been interpreted to mean that the proteolytic activity is the most essential function [240].

The arrangement of the m-AAA- and i-AAA-proteases in the inner mitochondrial membrane with active sites on opposite sides equips the mitochondria with the pro-

teolytic machinery for degrading integral membrane proteins with domains on either side. It has been pointed out that the complete degradation of such targets ultimately requires that the transmembrane region(s) also become exposed to the proteases. The chaperonelike activity of the m-AAA protease may be an essential function for this process [235].

Protein turnover in the cytosol was recognized several decades ago. Detailed mechanisms have become apparent only more recently. Numerous functions can be attributed to the process, for example: 1) quality control by removal of defective peptides; 2) control of gene expression in response to diverse signals; and 3) regulation of cell cycle progression. As distinct organelles with topologically separate compartments, mitochondria require their own set of proteases to achieve some of the same objectives.

#### 4.9 UNSOLVED PROBLEMS

The length of this chapter is testimony to the enormous progress which has been made in understanding the biogenesis of mitochondria, their genes and gene expression. The relative simplicity and economy of the metazoan mitochondrial genome is not the rule, and many challenges remain in the detailed understanding of the gene content and organization of mtDNA in plants, fungi, trypanosomes, and many other organisms. Molecular taxonomy is a fertile area. It remains to be seen whether completely novel and unorthodox phenomena such as the RNA editing process in Protozoa will be discovered in the future.

A major challenge continues to be to understand the interaction between the nuclear genome and the mitochondrial genome. The coordinate synthesis of many proteins both inside and outside may not be reducible to common, shared promoter elements in nuclear genes. The organ and tissue specific up or down regulation of mitochondrial capacity for OXPHOS constitutes an important problem which will be illuminated again in the discussion of mitochondrial diseases of humans (Chapter 7). Interesting problems are presented by altered energy metabolisms during different developmental stages of even relatively simple eukaryotes.

Mitochondrial DNA replication uncoupled from nuclear DNA replication in non-proliferating, differentiated cells is invoked to explain a number of observations, particularly changes in heteroplasmy and the accumulation of mutations in somatic cells, but is poorly understood. Even more of a puzzle is the postulated "bottleneck" in mitochondrial DNA replication in oogenesis to explain significant changes in heteroplasmy in one generation (see Chapter 7).

The questions related to RNA processing and/or editing are coming into focus. Details will clearly be added in the future.

The translation of mitochondrial mRNA also presents problems yet to be solved. There is still no *in vitro* system for translation of mt mRNA with constituents made exclusively from mitochondrial extracts. Initiation of translation on extremely short 5' untranslated regions of the transcripts is clearly very different from the situation in the cytosol. How many initiation factors are involved? Related to this may be the control of the differential stability of some of the transcripts.

While great strides have been made in elucidating the machinery for mitochondrial protein import, significant gaps remain in our understanding of the next stage. Simple soluble proteins may be refolded with the help of chaperones and released into the matrix as active enzymes. However, the assembly of the multisubunit complexes of the electron transport chain and their association with co-factors and metal ions (especially the nonheme Fe-S clusters) presents a still formidable problem. How sequential and interdependent is the process? What type of subcomplexes can assemble? How and when do integral membrane proteins made in the matrix interact with imported subunits? A full appreciation of the problem will be developed in Chapter 5, when the composition and structure of these complexes are presented in more detail.

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## MITOCHONDRIAL ELECTRON TRANSPORT AND OXIDATIVE PHOSPHORYLATION

### 5.1 HISTORICAL INTRODUCTION

The earliest history of research on mitochondria is presented in Chapter 1. Some far-sighted conclusions and very intelligent guesses made during the first half of this century turned out to be ahead of their time but could not be proved with the technology of the time, or were not pursued. From the biochemist's point of view a major milestone was the development of methods for the reproducible isolation of intact mitochondria. Biochemical reactions unique to mitochondria could therefore be studied for the first time, and it could be established that the reactions of the Krebs cycle and fatty acid oxidation were major or even exclusive pathways in mitochondria. Thus, the pathways and cycles leading to the complete oxidation of carbon compounds to carbon dioxide were elucidated, adding substance and detail to the conjecture already expressed by Lavoisier in 1789 that living organisms were slow combustion engines burning carbon compounds and hydrogen to CO<sub>2</sub> and water.

This fire stolen from heaven, this torch of Prometheus, does not only represent an ingenious and poetic idea, it is a faithful picture of the operations of nature, at least for animals that breathe; one may therefore say, with the ancients, that the torch of life lights itself at the moment the infant breathes for the first time, and it does not extinguish itself except at death. (Lavoisier, 1862, Vol.II, pp. 691–692, quoted in Reference 1)

In a celebrated experiment with a few forgivable flaws Lavoisier and Laplace demonstrated that the amount of heat released per volume of CO<sub>2</sub> produced was equal in the combustion of charcoal and in the respiration of a guinea pig. Combustion was believed then to take place in the lungs, and mitochondria were not yet on the horizon.

By 1807 Lazzaro Spallanzani's findings had been translated and publicized that carbon dioxide can be formed in a large variety of animal tissues, but for some time afterward the lungs were thought of as specialized combustion engines. Later on in the nineteenth century the blood became the favored site of respiration.

We should remind ourselves that thermodynamics was not fully developed until many decades later. What eventually became the first law of thermodynamics was first formulated by a physician, Robert Mayer, in 1842, reflecting on Lavoisier's theory about animal heat being derived from a combustion process, and blood entered the considerations because of the observations of color changes associated with heat production, and oxidation. In a later article Mayer correctly surmised that "blood corpuscles play the role of an oxygen carrier," but his ideas were not widely accepted until about 1875 when the role of hemoglobin in blood had been discovered and respiration in other tissues had been convincingly demonstrated. For example, one of the most dramatic demonstrations was to observe respiration in a frog whose blood had been replaced by a saline solution (recorded by E. Oertmann in 1877). James Joule provided a firm experimental foundation for the first law of thermodynamics in his celebrated experiments during 1843–1849. These studies inspired Helmholtz, a physiologist studying fermentation, to become a physicist and to write his memorable and influential article "Über die Erhaltung der Kraft" in which he clearly enunciated the idea of chemical potential energy released from respiration-combustion, and the conservation of this energy in the form of heat and mechanical energy. Assuming that mechanical energy (by muscles) was negligible, and in ignorance of the energy consumed by biosynthetic reactions and many other subcellular processes, he concluded that combustion of nutritional components yields the same quantity of energy of heat as that released by the animals.

The history of physiology in the late nineteenth century is full of illustrious personalities and their struggles with understanding blood and oxygen and combustion in animals. Two extensive quotes will suffice to illustrate the issues. In a long battle with opposing views E. Pflüger wrote in 1872:

Here lies, and I want to declare this once and for all, the real secret of the regulation of the oxygen consumption by the intact organism, a quantity determined only by the cell, not by the oxygen content of the blood, not by the tension of the aortic system, not by the rate of blood flow, not by the mode of cardiac action, not by the mode of respiration.

At about the same time (1878–1879) the famous French physiologist C. Bernard wrote:

... it is not in direct combustion that this gas (oxygen) is used. The usual formula repeated by all the physiologists that the role of oxygen is to support combustion is not correct. ... It is quite certain that this gas is fixed in the organism and that it thereby becomes one of the elements of organic structure or creation. But it is not at all through its combination with the organic matter that it incites vital function. Upon contact with the tissues, it makes them excitable; they cannot exist without this contact. It is therefore as an agent of excitation that it would participate intimately in most of the phenomena of life.

A more detailed account of the history of intracellular respiration can be found in the fascinating book by J.S. Fruton [1]. By the 1950s it was perfectly clear from a

basic understanding of chemical thermodynamics that the combustion of sugars and fats to  $\text{CO}_2$  was accompanied by the release of a substantial amount of energy, some of which was used to maintain our body temperature, but much of it interconverted into a chemical form of “free energy” to be used to drive biosyntheses and physiological reactions. The question was *how*?

The early decades of this century led to the important recognition that iron played a central role in oxygen consumption and respiration, and in 1924 O. Warburg was able to formulate the theory that

... molecular oxygen reacts with divalent iron, whereby there results a higher oxidation state of iron. The higher oxidation state reacts with the organic substance with the regeneration of divalent iron. ... Molecular oxygen never reacts with the organic substance.

In a remarkable paper Warburg defined the respiratory enzyme (*Atmungsferment*) as “... the sum of all the catalytically active iron compounds present in the cell.” The jump from the *Atmungsferment* to the definition of the mitochondrial electron transport complexes I–IV took almost another forty years. In the 1880s, cytochromes were first identified by C.A. MacMunn as histohematin and myohematin. Their role in the respiratory chain was, however, not appreciated until 1925, when Keilin placed them in the appropriate context, although Warburg continued to have doubts about the intracellular function of cytochromes. The objection was sustained on the one hand by Warburg’s attachment to the notion of the *Atmungsferment* as a single enzyme. Another good reason for Warburg’s resistance was that he had found that oxygen uptake by yeast was inhibited by carbon monoxide, and therefore cytochrome could not be the *Atmungsferment*, since cytochrome had not been discovered (in 1927) to react with carbon monoxide. It took another twelve years for Keilin and Hartree to demonstrate the existence of a CO-sensitive cytochrome absorbing near 600 nm, and its spectroscopic properties were identical to those of Warburg’s *Atmungsferment* complexed to CO. Keilin’s [2] detailed personal history of the evolution of ideas about respiration and the role of the cytochromes is highly readable.

We should remind ourselves that all these studies were carried out with whole-animal tissues by spectroscopic techniques, since mitochondria had not been isolated, and the solubilization of the cytochromes, with the exception of cytochrome c, had not been achieved.

There was a widespread assumption for some decades that all the oxygen consumed during respiration was accounted for in the carbon dioxide produced, and the hydrogens of carbohydrates appear to have been temporarily ignored by many. Again to quote C. Bernard (1876):

Later, more precise studies having shown that all of the oxygen was not represented in the carbonic acid, it was supposed that the surplus was used to burn hydrogen and form water. But, as noted by M.M. Regnault and Reiset, that is a gratuitous hypothesis. It has no reason for existence except to explain a deficit, itself hypothetical.

Experiments started primarily by H.O. Wieland around 1912 drew attention to the activation of hydrogen by palladium black, and biochemists were made aware that the



conversion of ethanol to acetic acid (in *Acetobacter*) was also an oxidation which could, however, proceed anaerobically, that is, in the absence of oxygen. Similarly, observations on putrefaction of organic materials had shown that hydrogen-rich compounds could be formed (methane, hydrogen sulfide), and it began to dawn on some that organic substances can be changed and cleaved by the action of water. From a number of pathbreaking discoveries on the interconversion of organic substances in various cell extracts enzymology was born. From a more limited perspective it also became clear that oxidative processes can also be considered dehydrogenations, which was shown elegantly by T. Thunberg in his work of 1917–1920. He prepared frog muscle as a suspension in an anaerobic environment and demonstrated that several organic acids (succinate, citric acid, glutamic acid, etc.) could be oxidized with the simultaneous reduction of methylene blue. These experiments and ideas culminated around 1924–1926 with the hypothesis that

hydrogen is transported, not directly from primary donors to oxygen, but by stages. It would appear that the path may in a given case be smoothened, and so the velocity of transport increased, by the intervention of a substance which can alternately act as an intermediate acceptor and donor. Such a substance acts therefore catalytically, as a carrier of hydrogen (Hopkins, 1926; Szent-Györgyi 1924) [1].

We can begin to see the glimmer of the idea of an electron transport chain, and the reader is invited to pursue the detailed lineage of ideas on this fascinating subject in the wonderful and scholarly account written by H.J. Fruton [1]. Some further milestones in the history of biochemistry and bioenergetics are presented in Chapter 6 on mitochondrial metabolism. We have reached the stage where it is clearly understood that organic acids and ultimately NADH are the hydrogen donors, and a “carrier” exists that separates these substances to be oxidized from molecular oxygen.

The discussion so far has focused on only one aspect of respiration as the driving force behind biological work, namely, the nature of the redox reactions and the participation of oxygen as the ultimate electron acceptor. Thermodynamic theory predicted that part of the free energy change resulting from these reactions would be in the form of the release of heat, as in ordinary combustions, but it became clear that many biochemical phenomena and reactions are endergonic and therefore required an input of energy in some form. What was the nature of the chemical free energy driving the biosynthesis of macromolecules, muscular contraction, or conduction of signals in the nervous system?

“Energy-rich” compounds were first isolated from muscle, leading to the characterization of ATP by K. Lohman, and of creatine phosphate, but the universal significance of ATP was not immediately recognized. In 1934, ATP was still considered a “coenzyme” in glycolysis. Many illustrious names in the history of biochemistry are linked to the clarification of ATP synthesis in the process of glycolysis, and the details of “substrate level phosphorylation” were beginning to be elucidated, culminating in the most influential articles by H. Kalckar and F. Lipman in 1941. These authors defined the concept of “high-energy phosphate bonds” and clarified the thermodynamics of phosphoryl-transfer reactions. On the other hand, when, in 1939, V.A. Engelhardt

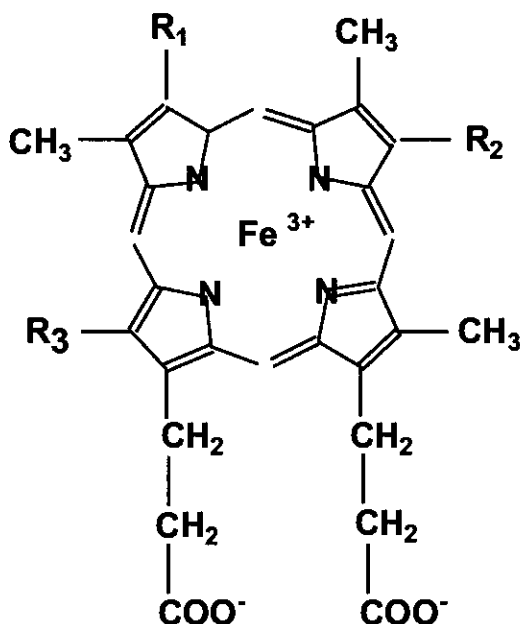
and M.N. Lyubimova identified myosin in muscle as an ATPase, and they and others linked the contractile process directly to the energy derived from ATP hydrolysis, the need for synthesizing ATP (or recycling it from ADP) became obvious. From this initial focus on muscle the general notion of ATP as the “energy currency” of all cells evolved, and within the next two decades the role of ATP in the synthesis of DNA, RNA and proteins and other macromolecules was rationalized and explained. It took significantly longer to fully understand how ATP was essential for the conduction of nerve impulses. Transmission of electrical signals required a membrane potential, and how such a membrane potential could be created could be understood only after the role of ATP in primary active transport was defined. It will become obvious from the discussion of the chemiosmotic hypothesis in the section below how the problem of ATP synthesis in mitochondria is conceptually related to active transport. The role of ATP in force generation and motility is appreciated now not only for muscle, but also for the dynein-based movement of cilia and flagella and kinesin-based movement along microtubules. The discovery of whole families of molecular motors (myosin, dynein, kinesin) using ATP has expanded the horizon even further. ATP is now recognized to be essential for protein targeting to subcellular compartments, membrane fusions in the secretory pathway, protein degradation by the proteasome, and last, but not least: What would signal transduction be without ATP, kinases, and phosphatases?

However, it is necessary to step back and dwell on another problem. Glycolysis and ATP production in the course of glycolysis are only part of the story, and every undergraduate is taught how much energy is “wasted” when lactate (or ethanol) is the end product of glycolysis (or fermentation) under anaerobic conditions. Converting pyruvate to  $\text{CO}_2$ , and to trap the released chemical free energy in the form of ATP became the next great challenge. It was decomposed into several problems: 1) the pathways of the breakdown of carbon compounds, solved brilliantly by H. Krebs with his formulation of the tricarboxylic acid cycle (Krebs cycle); 2) the oxidation of NADH and succinate by the mitochondrial electron transport chain; and 3) the coupling of electron transport to ATP synthesis (oxidative phosphorylation), culminating in the “chemiosmotic hypothesis” of P. Mitchell.

## 5.2 ELECTRON TRANSPORT CHAIN

### 5.2.1 Biochemical Components

The only component of the electron transport chain (ETC), which was successfully purified early on (1930s and 1940s), was cytochrome c, since it could be easily solubilized. The purified protein was further characterized as consisting of a peptide (~13 kDa), the apoprotein, and a heme linked covalently to two cysteine side chains in the peptide (Figures 5.1, 5.2). Because of the ease of its purification it could be obtained readily from many different species. Furthermore, its small size invited efforts to determine the amino acid sequence long before cloning and DNA sequencing made it a routine. In turn, sequence comparisons and other characteristics, as well as its ubiquitous distribution in living organisms made it a favorite for molecular evolutionists.



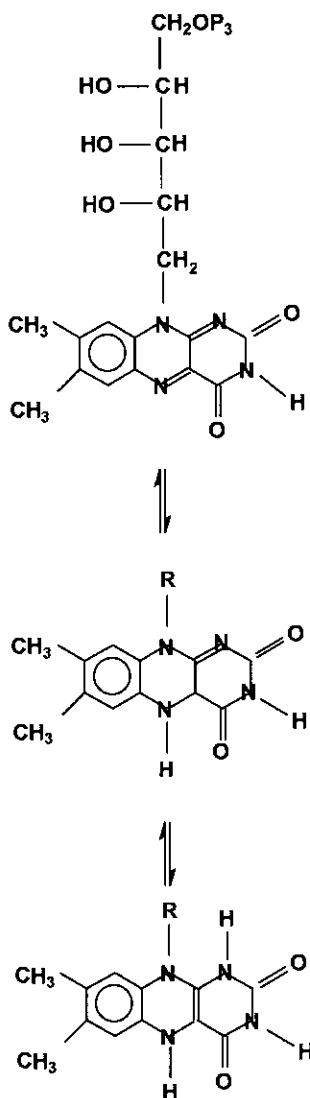
**Figure 5.1** The basic structure of a heme prosthetic group.

It is sobering to learn that in 1871 E.R. Lankester expressed the idea that “The chemical differences among the various species and genera of animals and plants are certainly as significant for the history of their origins as the differences in form” [1, p. 314]. The first phylogenetic trees constructed from amino acid sequence comparisons were made by E. Margoliash and M.O. Dayhoff, and those of cytochrome *c* were very prominent among them.

When mitochondria were finally identified as the “powerhouse of the cell,” it became easier to look for the “carrier” between organic hydrogen and oxygen, but almost another fifteen years elapsed before the “carrier” was resolved into a series of complexes forming the electron transport chain, with ubiquinone and cytochrome *c* acting as electron carriers between the complexes. It is difficult to do justice to all the discoveries and to define in a simple, linear progression the chain of ideas that contributed to the picture of the electron transport chain as presently found in elementary biology textbooks. As noted earlier, cytochromes were characterized at first mainly by spectroscopic methods, and eventually their chromophore was established as a heme, bound to proteins, and thus localized in a microenvironment, which determined its spectroscopic properties and its redox potential. Type *c* cytochromes have covalently bound heme, while type *a* and *b* cytochromes have hemes in a noncovalent attachment. Parallel studies of heme in soluble hemoglobin were of course most valuable.

Another major breakthrough belonging to the earlier phase was O. Warburg’s discovery of the “old yellow enzyme” (1932–1933), the identification of the coferment





**Figure 5.3** The structure of flavin mononucleotide showing the flavin in the fully oxidized form (**top**) and in the fully reduced form (**bottom**).

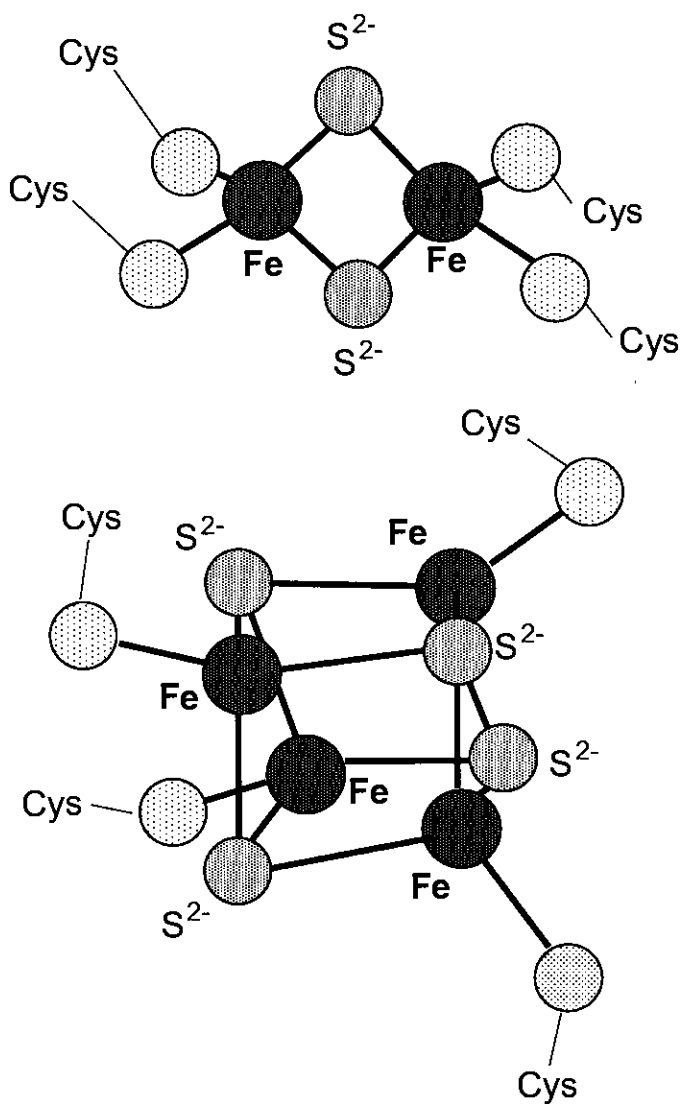
Many individuals have contributed to the precise definition of this electron transport chain, and the reader is referred to the monograph of W. Wainio [5] for an authoritative and detailed review of the literature up to about 1970. Key contributions were many, but the experiments from the B. Chance laboratory using dual-beam, dual-wavelength spectrophotometers stand out. They contributed not only to a further definition of the spectroscopic properties of the cytochromes, but kinetic and inhibitor

studies resolved the direction of electron flow, defined coupling sites, and further supported earlier thermodynamic calculations by E. Ball and F. Lipman showing that the free-energy change associated with the oxidation of NADH is sufficient for the generation of three ATPs. Oxidation of succinate was predicted to yield only two ATPs. The enzyme succinate dehydrogenase (SDH) was found to be the only enzyme of the Krebs cycle that was insoluble and attached to the inner mitochondrial membrane, coupling the Krebs cycle to the electron transport chain. Thus, succinate can reduce the flavin in SDH directly. The recognition of several distinct flavoproteins was important, as the above simple scheme emphasizes the distinction between FMN and FAD.

The advent and application of more sophisticated spectroscopic techniques in the 1950s and 1960s brought a final major discovery about electron transport with proteins. Electron spin resonance (ESR), now referred to as electron paramagnetic resonance (EPR), which detects the spins of unpaired electrons in a transition metal ion, was applied to whole heart tissue or mitochondria in pioneering studies by H. Beinert [6], leading to the discovery of a novel kind of metalloprotein containing iron in a nonheme form. The iron is found in so-called iron-sulfur clusters, and their diversity and abundance in the ETC was at first quite a surprise [6]. They had escaped detection because the optical properties, measurable by spectrophotometry, were obscured by the strong absorption of the cytochromes. The structures of many such clusters have since been characterized (Figure 5.4). In addition to their role in electron transfer, their structural versatility has been adapted in a large variety of proteins to "accept, donate, shift and store electrons." Cluster construction as well as cluster interconversions by ligand exchanges and oxidative degradation constitute interesting biological reactions. The literature on the subject has become vast, with whole books on the subject appearing periodically. A very insightful and comprehensive review on recent developments has been written by Beinert and colleagues [7].

The iron is coordinated with two to four cysteine side chains from the polypeptide, and in addition the cluster generally contains an equal number of sulfide ions ( $S^{2-}$ ); these sulfides are acid labile and released at low pH as hydrogen sulfide ( $H_2S$ ), a biochemical diagnostic for the presence of such clusters. In most clusters each iron is coordinated to a total of four S atoms in a roughly tetrahedral arrangement. The [Fe-S] cluster, with a single Fe linked to four cysteine residues (and no sulfide), has been found only in bacteria. Eukaryotic proteins have been found to contain the clusters [2Fe-2S], [3Fe-4S], and [4Fe-4S]. The iron atoms in each cluster form a conjugated system, and instead of a single iron forming a  $Fe^{2+}/Fe^{3+}$  redox couple, the entire cluster can lose or gain electrons. For example, the [4Fe-4S] cluster contains one Fe(II) and three Fe(III)'s in the oxidized form and two each of Fe(II) and Fe(III) in the reduced form. In general the cluster can be represented by  $[mFe-nS]^x$ . Each cluster has its characteristic redox potential, and with unpaired electrons each cluster also has a characteristic EPR spectrum (when  $x = 1$  or  $x = 3$ ). EPR can thus detect the gain or loss of electrons at each cluster.

The presence of such clusters in the mitochondrial ETC provided another mechanism for the passage of an electron through a protein. It should be mentioned that Fe-S clusters were eventually also found in many proteins unrelated to the mito-



**Figure 5.4** Structure of the [2Fe-2S] and [4Fe-4S] iron-sulfur clusters.

chondrial electron transport chain. Not surprisingly, they were found in proteins involved in photosynthesis and nitrogen fixation, but also in soluble proteins such as xanthine oxidase, ferredoxins, and numerous hydrogenases in bacteria. Not all Fe-S centers are devoted to electron transfer, but they may also serve in the stabilization of the tertiary structure of proteins. A particularly interesting example is the protein binding to the iron-responsive element (IRE) in certain mRNAs. This protein has been identified as the cytosolic aconitase, and iron binding involves the formation of

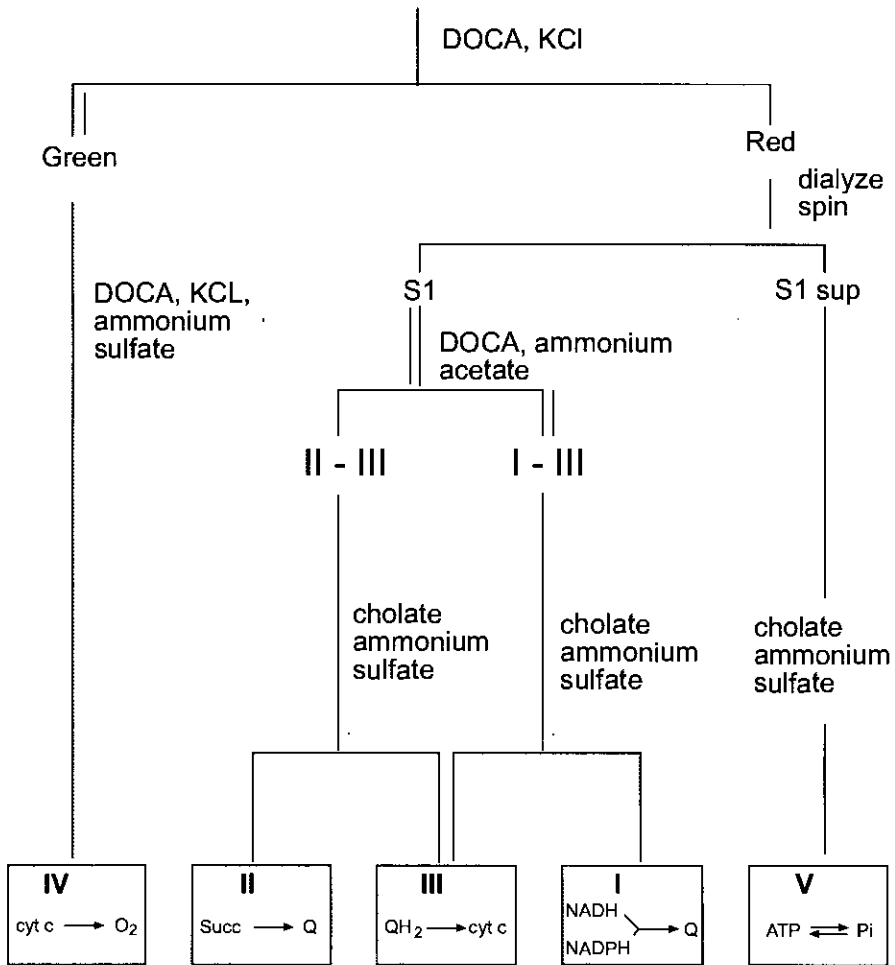
an Fe–S center accompanied by a conformational change in the protein. Disassembly of the center in the absence of iron leads to a conformation with high affinity for the IRE. In the mitochondrial matrix the Fe–S center in the same protein is active in electron displacements during the interconversion of citrate to isocitrate. In mitochondrial membranes, the next challenge was to localize them and place them in a position in electron transport relative to the flavins, ubiquinones, and cytochromes. The original discoveries by Beinert and Sands [6] were made in the NADH and succinate dehydrogenases (see below), and a third such center was found in what is now known as Rieske's protein in the cytochrome b–c<sub>1</sub> complex (complex III), named after the original discoverer [8]. More centers in mitochondria were discovered when EPR spectroscopy was carried out at very low temperatures with a significant gain in resolution, but their precise position within the overall ETC could not be determined without further purification.

### 5.2.2 Physical Separation of the Complexes of the Electron Transport Chain

A hotbed of biochemical research on the electron transport chain was the laboratory of D. Green at the Enzyme Institute in Madison, WI, from which many of the pioneers in the field emerged in the late 1950s and early 1960s. The stage was set for the pioneering breakthroughs achieved by Y. Hatefi and his collaborators in the late 1960s [9–11]. Starting with enormous quantities of beef heart, mitochondria were isolated and purified in correspondingly large amounts and subjected to systematic solubilizations and fractionations (Figure 5.5). It should be recalled that our concepts of membrane structure and membrane proteins were rudimentary at that time. While other laboratories had attempted to isolate individual cytochromes, Hatefi sought to isolate proteins or protein complexes that would be functionally intact and capable of carrying out individual redox reactions that had been associated with respiration. He exploited the observation that coenzyme Q (ubiquinone; Figure 5.6) and cytochrome c could be easily and reversibly removed from mitochondria by an appropriate solvent or salt extraction, respectively, and the suggestion that these two compounds were mobile electron carriers: ubiquinone was found to act between complexes I or II and III, and cytochrome c between complexes III and IV. Based on these insights, assays for the function of each individual complex could be developed. Complex I oxidized NADH with ubiquinone as electron acceptor; the same electron acceptor was used in the oxidation of succinate by complex II; a reduced complex III was able to donate electrons to cytochrome c; reduced cytochrome c could be oxidized by complex IV with oxygen as electron acceptor. Finally, complex V was assayed as an ATPase. The devised scheme allowed the isolation of all five complexes from the same batch of mitochondria. For the first time it became apparent that each complex consisted of multiple peptides, and required the presence of lipids for solubilization and stabilization.

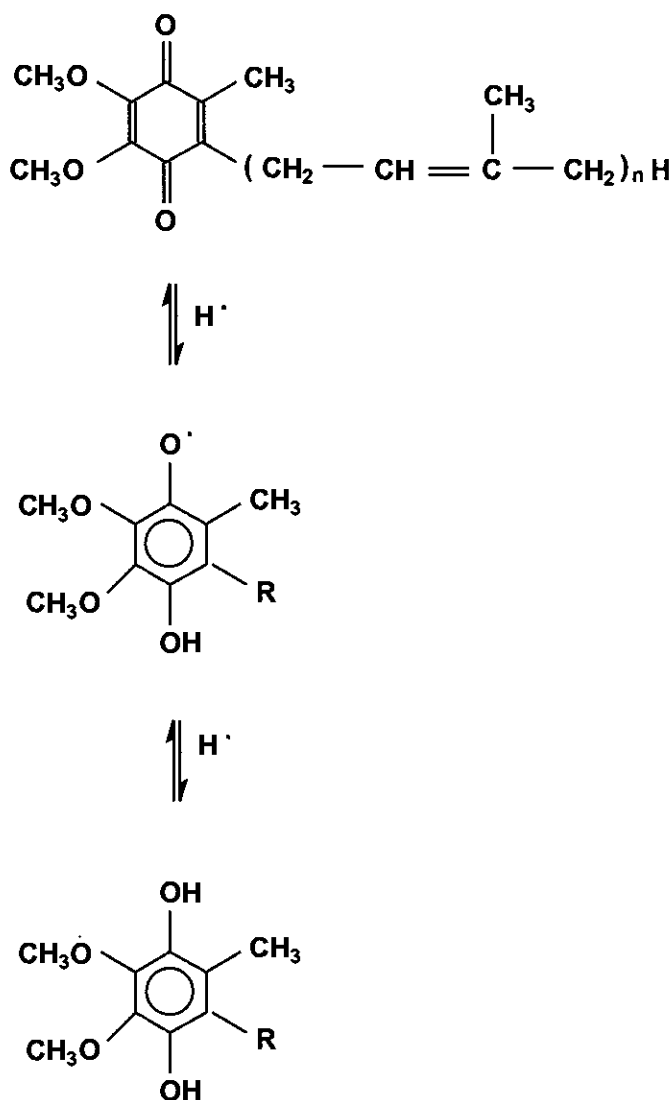
These isolation procedures provided relatively pure complexes with which more detailed biochemical and biophysical analyses could be performed. The earliest data were confirmed and refined over the years, and there is no reason to dwell on some of the uncertainties which were resolved with time. The most challenging problem was to establish very precise stoichiometries in the various complexes with respect to





**Figure 5.5** An outline of the purification scheme devised by Hatefi to obtain all five complexes from the inner mitochondrial membrane of bovine heart. The complexes are assayed and defined by the reactions shown in the boxes at the bottom. See Reference [9] and previous papers cited there.

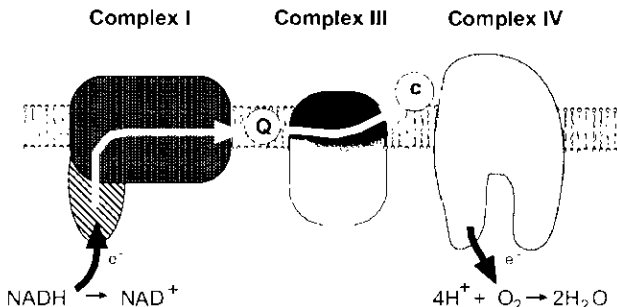
flavin content (complexes I and II), iron content, and acid-labile sulfide (complexes I, II, and III). Another issue was to determine the number of polypeptides which were specifically associated with each complex, and, while this number was settled quite quickly for complexes II to V, the peptide composition of complex I continued to be a significant challenge over the years. Initial estimates of over 10 polypeptides [9] were revised to approximately 25 polypeptides based on two-dimensional gel electrophoresis [12], to the most recent analysis by high-resolution chromatography and sequencing yielding at least 41 peptides [13]. As discussed elsewhere in this book, all complexes except complex II were shown to contain peptides made in the mitochon-



**Figure 5.6** Structure of ubiquinone (coenzyme Q) in the oxidized, partly reduced (free radical) and fully reduced form. The number of isoprenoid units in the long hydrophobic tail may vary slightly in different organisms.

drial matrix, and their number and identity was settled after the complete human mtDNA sequence had been published and analyzed [14,15].

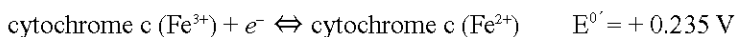
In the early 1970s the mitochondrial electron transport chain in mammalian mitochondria had taken on its present form which is schematically represented in Figure 5.7. Indeed, in 1975 each complex could be presented only as a box, or by some other



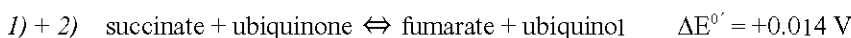
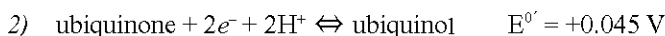
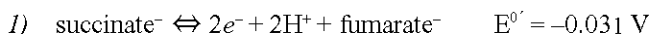
**Figure 5.7** Schematic representation of the electron transport chain from NADH to oxygen. Complexes I, III, and IV are shown, as well as the mobile carriers, Q, and cytochrome c.

more or less rounded, asymmetrical shape. For the biochemist, structural biochemist and biophysicist the challenge was to determine the interaction of the various subunits within each complex, and to orient these peptides relative to the inner and outer surface of the inner mitochondrial membrane. The final goal was to determine the crystal structure of each complex and to understand 1) electron transport through the complex, 2) proton translocation across the inner membrane which is coupled to electron transport, 3) the site and mechanism of action of specific inhibitors, and 4) most recently, the effect of specific missense mutations associated with human mitochondrial diseases (Chapter 7). The inner mitochondrial membrane, although exceptionally protein-rich, is nevertheless still a fluid membrane [16,17], and the assumption of each complex being distinct but connected functionally (for electron transport) to the next via mobile carriers (ubiquinone or cytochrome c) is a valid one. The complexes diffuse in the plane of the membrane and they probably collide, but a direct electron transfer during such collisions is not observed. It appears that in bovine heart mitochondria each complex is present in a stoichiometric ratio to the other complexes : 1 complex I, 2 complex II, 3 complex III, 6–7 complex IV, and 4–5 complex V (Hatefi, personal communication), but the data may be too limited for a broader generalization. Furthermore, as discussed in Chapter 3, there is probably a developmentally regulated and tissue-specific expression of the genes encoding the electron transport chain, which adapts the capacity of the mitochondrial oxidative phosphorylation system to the requirements of specific tissues and cells.

From a thermodynamic and electrochemical perspective each step in the electron transport chain is associated with a free-energy change, and each component undergoes a cycle of oxidation and reduction reactions, that is, it represents a redox couple, or half-reaction. The properties of such a redox couple are conveniently described by the standard reduction potential, a quantity that can be easily related to an associated free-energy change. For example, for the mobile cytochrome c the following describes the half-reaction and the corresponding standard reduction potential:



A complete reaction consists of the sum of two half-reactions:



One can calculate the free energy change for this oxidation reduction reaction from the relationship

$$\Delta G = -n\mathcal{F} \Delta E$$

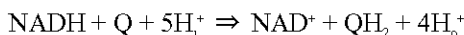
where  $\mathcal{F}$  is the faraday ( $1\mathcal{F} = 96,494 \text{ cal/mol}$ ). The standard reduction potentials are defined relative to the hydrogen half-reaction under standard conditions ( $\text{pH} = 0$ ,  $25^\circ\text{C}$ ,  $\text{H}_2(\text{g})$  at 1 atm), and the prime superscript in the above symbols indicates that the values are for biological systems operating at pH 7.

It is informative and instructive to consider the electron transport chain from the point of view of an electron finding itself at a high potential (in NADH), dropping through a series of intermediate stages to its final destination, oxygen. This reaction, with reference to the various half-reactions and their associated standard reduction potential, is shown in Figure 5.8; it is apparent from such a representation that there is not a very large drop between succinate and ubiquinone, and the free energy change may be too small for proton pumping (see Section 5.4). The most substantial change in potential occurs in the oxidation of NADH by ubiquinone, and in the last step, reduction of oxygen to water. These steps are therefore virtually irreversible.

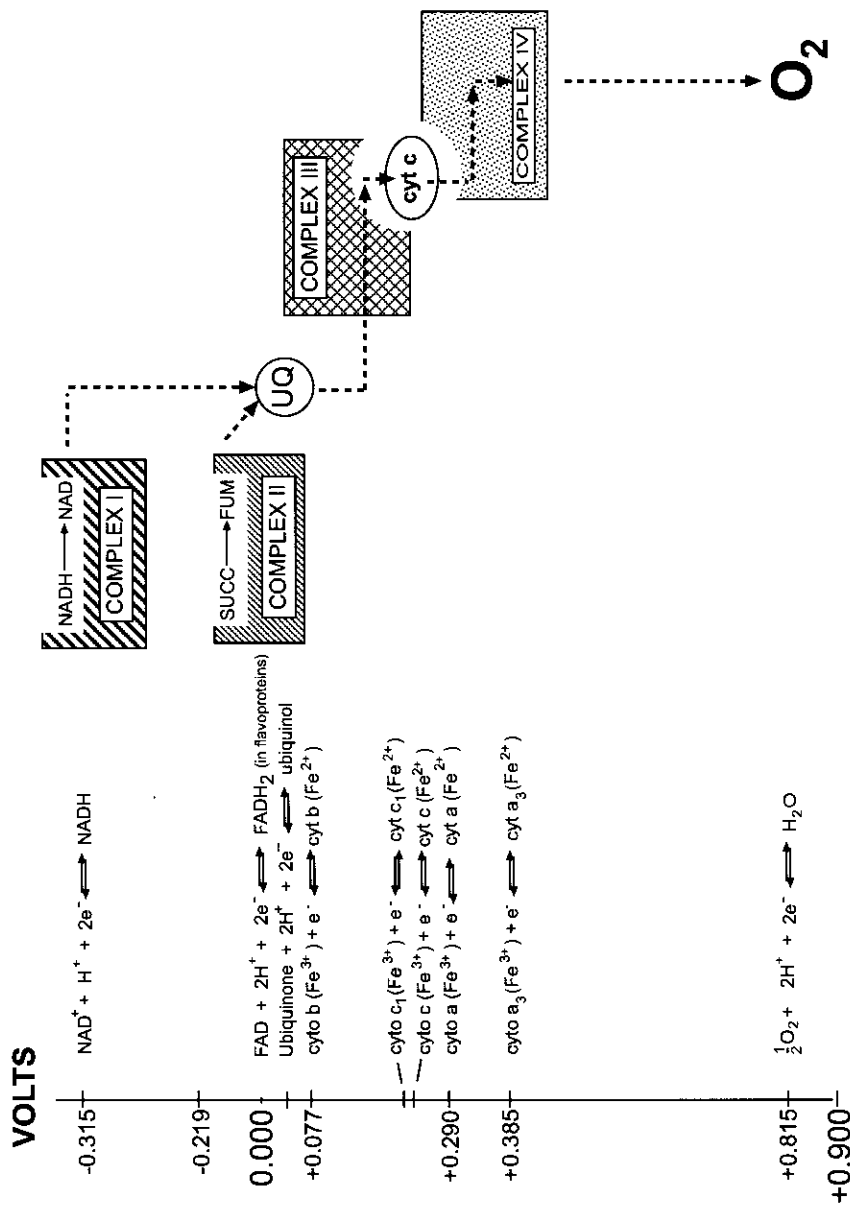
In the following, each complex will be discussed in turn, with an emphasis on composition, structure and function. Some comparative data will also be introduced to emphasize similarities and differences of these complexes in mitochondria of organisms from a broader phylogenetic range. A discussion of complex V will be deferred until after the section on the chemiosmotic hypothesis

### 5.2.2.1 Complex I

Complex I is properly referred to as NADH-ubiquinone oxidoreductase with the inclusion of the substrates in the name. The overall reaction catalyzed by this complex can be described as follows:



Q and  $\text{QH}_2$  refer to the oxidized and reduced form of ubiquinone, respectively. Of great significance is the number and subscript designation of the protons on either side of the equation, which will be made fully apparent in the section to follow. Here it suffices to state that the reaction is accompanied by the net transfer of four protons from the inside (matrix side) to the outside (intermembrane space). This apparently simple



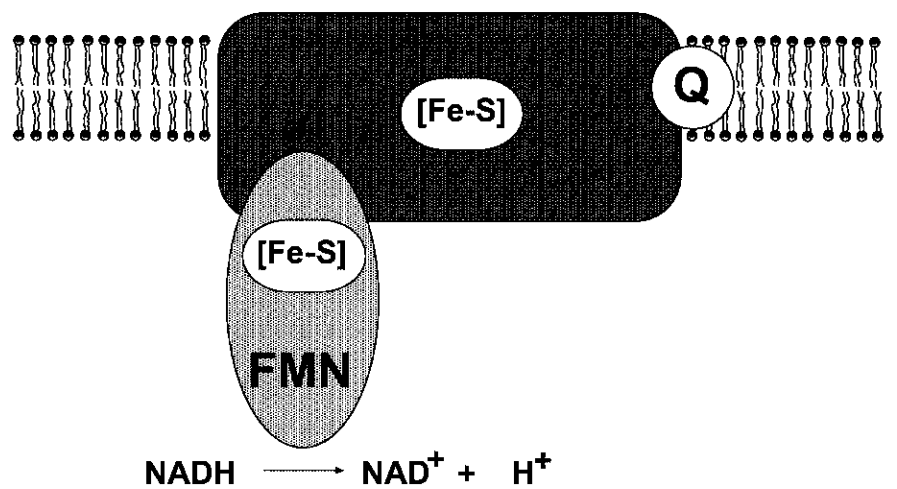
**Figure 5.8** Half-reactions, standard reduction potentials, and the electron transport chain. (The parameters were obtained from Table 15-4 in Reference 17a, with permission.)

reaction requires a complex with at least 42 peptides (~900 kDa) in mammalian mitochondria. A similar complex in the membrane of prokaryotes has an estimated molecular mass of ~520 kDa, with only 14 peptides. Its analysis in other eukaryotic organisms is incomplete, but curiously, common yeasts such as budding and fission yeast use a completely different enzyme for electron transfer from NADH to ubiquinone which does not pump protons out of the matrix. Thus, another microorganism, *Neurospora crassa* has become a model system for the study of this complex in preference to a mammalian source (beef heart), particularly when genetic studies and the use of mutants became powerful tools in the analysis [18]. Seven of the subunits in mammals and *Neurospora* are encoded by the mitochondrial genome. Nuclear mutations affecting the activity of the mammalian complex I have been isolated in cells in tissue culture [19], and some of these mutations have been mapped on the mammalian X chromosome [20,21]. The cDNA encoding the small MWFE/NDUFA1 peptide has been shown to complement the defect in these complex I-deficient Chinese hamster cells (Scheffler et al., in preparation). Primarily in the laboratory of J.E. Walker [13], 34 nuclear encoded subunits of the bovine complex have been cloned and sequenced as cDNAs. The recent interest in human diseases resulting from complex I deficiency [22] has prompted the cloning of many of the corresponding human cDNAs or genes, and their mapping on various chromosomes [recent summary in Reference 22]. Both nuclear mutations and mitochondrial mutations have been found in human patients (see Chapter 7 for further discussion).

The overall, low-resolution structure of the complex has been derived from electron microscopy, and it can be described by an L shape, with the long arm integrated into the inner membrane and the short arm extending into the matrix (Figure 5.9). A chemical analysis of the complex has revealed the presence of a flavin mononucleotide (FMN) and up to seven nonheme iron sulfur clusters, of which only four or five have been resolved and characterized by EPR spectroscopy. The short arm of the L can be described functionally as an NADH dehydrogenase, and it contains the FMN cofactor as well as at least four of the Fe-S clusters. The binding site for the substrate NADH is found on this subcomplex exposed to the mitochondrial matrix. This arm can also be referred to as the peripheral arm. The long, membrane embedded arm functions as the ubiquinone hydrogenase. The seven hydrophobic subunits encoded by mtDNA are part of the long arm.

It is believed that the mammalian, plant and the *Neurospora* complexes have similar structure, and therefore there will be no distinction in the following discussion, unless specific distinctions are indicated. The proton-pumping NADH:ubiquinone oxidoreductase in prokaryotes has fewer peptides [23], and it is thought that the 14 peptides found in *E. coli* represent the minimal number structurally and functionally necessary. As found in other complexes, the additional peptides in higher eukaryotes may represent scaffolding and assembly factors not directly involved in electron transfer and proton transfer. A comparison of peptide compositions in different organisms, including *E. coli*, is presented in Table 5.1. It is adapted from a recent review of the complex I in plants [24].

Since the additional 28 peptides found in the mammalian complex have no counterpart in a fully functional bacterial complex, their role in the mammalian complex



**Figure 5.9** Highly simplified representation of complex I, the NADH-ubiquinone oxidoreductase. The entire complex has more than 40 polypeptides. The number of iron-sulfur centers is greater than indicated in the figure.

remains puzzling. Some of them are also unusual in another respect: although they must have a signal sequence for mitochondrial import, there is no cleavable N-terminal leader sequence. A stimulating discussion of the organization and evolution of structural elements within complex I has been presented by Fine1 [25]. It expands on

**TABLE 5.1    Nomenclature and Comparison of Complex I Subunits/Genes in Bacteria, Mammals, and Plants**

| E. coli | Mammals  |       | Plants     |       |
|---------|----------|-------|------------|-------|
|         | nDNA     | MtDNA | nDNA       | MtDNA |
| nuoH    |          | ND1   |            | nad1  |
| nuoN    |          | ND2   |            | nad2  |
| nuoA    |          | ND3   |            | nad3  |
| nuoM    |          | ND4   |            | nad4  |
| nuoK    |          | ND4L  |            | nad4L |
| nuoL    |          | ND5   |            | nad5  |
| nuoJ    |          | ND6   |            | nad6  |
| nuoD    | 49kD     |       |            | nad7  |
| nuoI    | TYKY     |       | 28.5kD     |       |
| nuoC    | 30kD     |       |            | nad9  |
| nuoB    | PSST     |       | 22kD       |       |
| nuoE    | 24kD     |       | (24kD)     |       |
| nuoF    | 51kD     |       | 55kD       |       |
| nuoG    | 75kD     |       | 75kD       |       |
|         | ~28      |       | Additional |       |
|         | peptides |       | peptides?  |       |

a preceding proposal that complex I evolved by the combination of modules of pre-existing domains of ancestral enzymes [see also Reference 26].

A genetic approach in *Neurospora crassa* has been particularly fruitful in the exploration of the assembly of this complex and of the distribution of peptides in the NADH dehydrogenase and the ubiquinone hydrogenase subcomplexes [18]. Effectively, gene knockouts can be achieved by site-specific integration of a hygromycin resistance marker, and the consequences of a missing subunit on assembly can be studied. The examination of a variety of different mutated genes has already provided a wealth of information. A 51-kDa peptide (*nuo51* gene) has been identified to bind the substrate NADH, and to contain the FMN cofactor and one [4Fe-4S] cluster (N-3). In its absence an almost complete but inactive complex I is assembled missing only the FMN and the Fe-S cluster N-3. The mutant *nuo21* is of special interest because in the absence of this peptide the peripheral complex is assembled completely, while assembly of the membrane arm is only partial. With this mutant as starting material, the peripheral subcomplex can be prepared and purified conveniently. It can oxidize NADH when ferricyanide is used as an artificial electron acceptor, and it even has a low NADH : ubiquinone oxidoreductase activity. The latter is insensitive to piericidin A and thus believed to be due to ubiquinone binding to a site on the peripheral arm, which is not normally exposed in an intact complex I. The purified peripheral subcomplex has 15 peptides (all nuclear encoded), and three Fe-S clusters (N-1, N-3, and N-4). Further work on this subcomplex is in progress, and it can be hoped that attempts to crystallize it will be successful, leading eventually to a high-resolution structure by x-ray crystallography. The same *nuo21* mutant has also been used to purify an intermediate in the assembly of the membrane arm. A similar approach is taken with bovine subcomplexes obtained by purely biochemical procedures.

*Neurospora* mutants defective in a nuclear encoded complex I peptide have a reduced growth rate, but their mitochondria are not significantly respiration-deficient. The presence of cytochromes and inhibition of respiration by antimycin and cyanide suggests a functioning electron transport chain downstream from ubiquinone, and the absence of a piericidin-sensitive complex I is overcome by an alternative NADH-ubiquinone oxidoreductase.

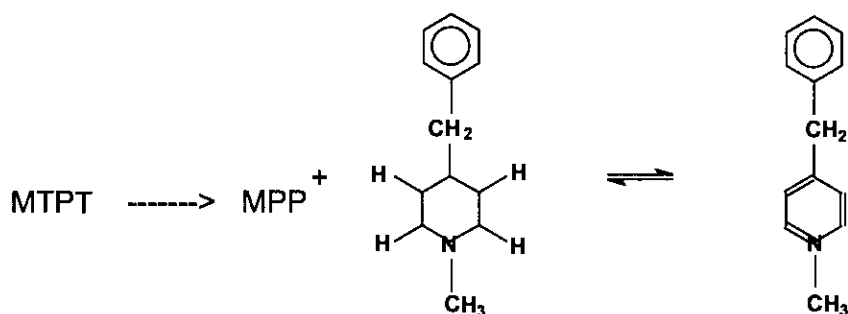
Since a genetic approach is not possible with the bovine complex, attempts to disrupt and solubilize portions of the complex I have made use of the chaotropic ion perchlorate or the detergent lauryldimethylamine oxide. With the use of perchlorate three fractions have been obtained, referred to as the flavoprotein (Fp), the iron-sulfur protein (Ip), and a hydrophobic complex. The Fp complex consists of three peptides, (51, 24, and 9 kDa, respectively), and further analyses have confirmed the largest peptide to bind NADH, and to contain the FMN and the [4Fe-4S] cluster. A second Fe-S cluster [2Fe-2S] is found in the 24-kDa peptide, while the 9-kDa peptide has no features or identifiable functions. The detergent splits the complex differently, yielding subcomplexes I $\alpha$  and I $\beta$ . The I $\alpha$  subcomplex appears to retain the capacity for transferring electrons from NADH to ubiquinone, while subcomplex I $\beta$  is an inactive portion of the membrane complex. However, this oxidation of NADH is insensitive to rotenone, and the ubiquinone acts as an artificial electron acceptor from an abnormal site on the subcomplex. Most of the individual peptides, either nuclear encoded or mi-



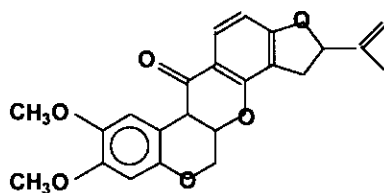
tochondrial, have been assigned to one or the other of these subcomplexes, but in view of the multitude of peptides a final picture of the arrangement of each of these peptides within the complex will probably not be available until the crystal structure is obtained.

Over the years the analysis of this complex and of its activity in mitochondria has been aided by the discovery of a variety of highly specific, naturally occurring inhibitors (Figure 5.10). The best known of these is rotenone, used as a fish poison; another is the antibiotic piericidin A. Some of these are being developed as potential insecticides and for various other pharmacological purposes [27]. As discussed elsewhere in this volume, the "designer drug" MPTP (1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine) can be converted to the active metabolite 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>) by monoamine oxidase and transported into dopaminergic neurons. At sufficiently high concentrations (mM) it is a specific inhibitor of complex I. This mechanism is thought to be responsible for the death of neurons in the substantia nigra and hence symptoms of parkinsonism in humans and experimental primates [28,29]. A large and diverse series of specific inhibitors of complex I, some occurring naturally and some synthetics, have been discussed and investigated systematically by Friedrich and colleagues [30]. On the basis of detailed kinetic analyses these authors were able to divide the inhibitors into two classes: class I inhibitors (piericidin A, annonin VI, phenalamid A2, aurachins A and B, thiangazole, and fenpyroximate [synthetic]) inhibit complex I in a partially competitive manner with respect to ubiquinone; class II inhibitors (rotenone, phenoxan, aureothin, and benzimidazole [synthetic]) act in a noncompetitive manner (Figure 5.10). None of the inhibitors inhibit the NADH-ferricyanide reductase, that is, the peripheral membrane arm of the complex extending into the matrix. Instead, all inhibitors appear to arrest the transfer of electrons from the high-potential iron-sulfur cluster (N-2) to ubiquinone. Since the Fe-S clusters have not been completely characterized in this complex, it is not possible to be more specific. However, photoaffinity labeling studies with a rotenone derivative have suggested that this binding site is close to the peptide encoded by the mitochondrial ND1 gene. A more recent investigation of rotenone binding raising several still controversial issues related to conformational changes within the complex has been published by Grivennikova and coworkers [31]. Specifically, these authors propose that one rotenone-specific site in complex I affects NADH oxidation by ubiquinone, while a second site is active in the ubiquinol-NAD<sup>+</sup> reductase reaction. This apparent violation of the principle of microscopic reversibility clearly deserves further clarification.

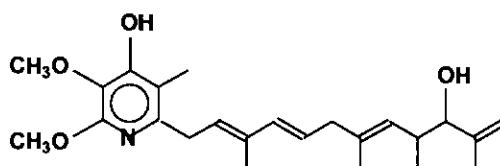
Detailed studies on the mechanism of inhibition by MPP<sup>+</sup> analogues by Singer and colleagues [32] have led to the interpretation that there are two MPP<sup>+</sup> binding sites, both of which must be occupied for complete inhibition. A "hydrophilic site" is defined by accessibility to relatively hydrophilic analogues, while a "hydrophobic site" may be less exposed to the aqueous environment. This interpretation is consistent with findings that the complex I can contain two ubisemiquinones. Miyoshi and colleagues [33] have reexamined this question with the help of a new series of *N*-methylpyridinium and *N*-methylquinolinium analogues, with the goal of finding a more potent inhibitor effective at lower concentrations, and perhaps more signifi-



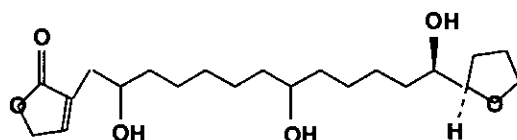
Phenoxan



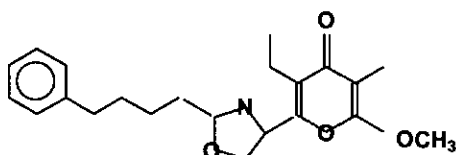
Piericidin A



Annonin VI

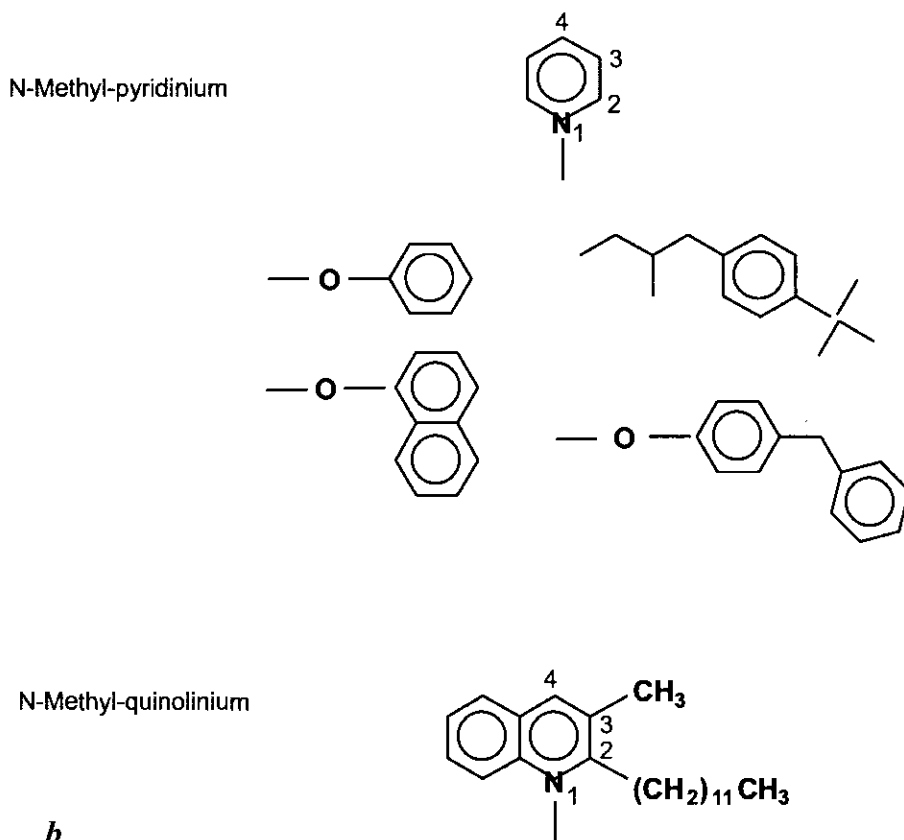


Rotenone



a

cantly, the aim of finding an inhibitor which exhibits selectivity for only one of the two sites. The results clearly support the two-site model, and significant progress toward achieving the second aim was also made. The presence of two ubiquinone binding sites is in good agreement with the acceptance of ubisemiquinone as an obligatory intermediate in the two-electron transfer from NADH to ubiquinone [34,35].



**Figure 5.10** **A:** Structures of specific inhibitors of complex I frequently used in the study of this complex. **B:** The compounds shown in *b* are *N*-methylpyridinium analogues in which various side chains can be substituted at positions 2, 3, or 4. (Modified from References 30 and 33.)

#### 5.2.2.2 Complex II

Complex II (succinate:ubiquinone oxidoreductase) consists of only four peptides and is thus the simplest of all the complexes of the ETC. The two largest peptides constitute the peripheral portion of the complex and function as the enzyme succinate dehydrogenase in the Krebs cycle. They are associated with the membrane through two integral membrane proteins, also referred to as the “anchor” proteins. Electrons from the oxidation of succinate to fumarate are channeled through this complex to ubiquinone. Thus, complex II links the Krebs cycle directly to the ETC [36–38]. A highly conserved and similar complex is also found as part of the electron transport system in bacterial membranes. The corresponding genes were first cloned in bacteria, and the bacterial systems (*Bacillus subtilis* and *Escherichia coli*)

have been very useful model systems in the study of the structure, function, and assembly of this complex. Excellent authoritative reviews have been written by several authors [39–41].

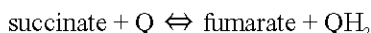
Cofactors and metal ions make up the additional components of complex II. A flavin is linked covalently to the largest peptide (70 kDa), yielding the flavoprotein subunit (Fp). Singer and his colleagues [42–44] established the chemical linkage in the early 1970s, and they also identified a histidine as the point of attachment. The timing and mechanism of this conjugation reaction was an open question until recently when the Fp gene (*SDHI*) was cloned in yeast. Conversion of His90 to Ser90 by site-directed mutagenesis prevented covalent attachment of FAD, but the assembly of the complex II in mitochondria was not affected [45–47]. It was clearly shown that flavin attachment to the normal Fp peptide occurred in the mitochondrial matrix, after the removal of the targeting sequence [45]. Additional experiments were interpreted to indicate that the enzyme responsible for this modification has to recognize a folded Fp peptide as one of its substrates, rather than a short peptide sequence surrounding the targeted histidine side chain. Nevertheless, this region of the peptide is highly conserved in evolution [see 48 for a review]. When complex II was assembled in yeast with a noncovalently bound flavin, the complex was active only as a fumarate reductase, not as a succinate dehydrogenase [47]. This result supports the idea that the covalent attachment of the flavin raises its midpoint potential to the level required for the oxidation of succinate [49].

The Fp subunit is intimately associated with the iron-protein subunit (Ip), made up of a peptide of 27 kDa containing three non-heme Fe–S centers, [2Fe–2S], [3Fe–4S], [4Fe–4S] [50]. Each has a characteristic redox potential, and together these centers serve in the electron transport through the subunit [39,44,51]. The Ip peptides from all species examined to date, and even from prokaryotes, have three highly conserved cysteine clusters (CI, CII, CIII) within the peptide. The first cluster is used to make the [2Fe–2S] center, and it resembles the [2Fe–2S] cluster also found in plant ferredoxins. The [4Fe–4S] center is made from the first, second and third cysteines of cluster II and the third cysteine of cluster III, while the [3Fe–4S] center is made from the first and second cysteines of cluster III and the fourth cysteine of cluster II. These deductions, from EPR studies made with the help of site-directed mutagenesis of bacterial *sdh* and *frd* iron-proteins, remain to be confirmed for eukaryotes. Thus, the assembly of the clusters depends on the secondary and tertiary structure of the Ip subunit rather than the local peptide sequence.

The Fp–Ip complex (SDH) can be dissociated from an isolated complex II by treatment with chaotropic ions as first shown by Hatefi and Davis [38]. It functions as a succinate dehydrogenase, when an artificial electron acceptor such as ferricyanide or tetrazolium is included in the assay, but cannot interact directly with ubiquinones. To reconstitute ubiquinone reduction, the SDH complex must be combined with the two integral membrane proteins of complex II,  $C_{II-3}$  and  $C_{II-4}$ , also referred to as QPs-1 and QPs-2. These “anchor” proteins are small peptides of molecular weight 15 and 12–13 kDa, respectively (in mammals). Only a few genes have been cloned, but sequence analysis suggests that each has three transmembrane segments, and an N-terminal domain extending into the matrix for interaction with the SDH enzyme [52,53].

Solubilization and reconstitution experiments, sequence analysis and hydropathy plots, and some earlier radiolabeling experiments by Merli and associates [54] suggest a structure for complex II shown in Figure 5.11. There is some analogy with complex I, but on a much simpler scale. The peripheral subcomplex (SDH) has the substrate binding site (succinate), the hydrogen acceptor (FAD), and 3 [Fe-S] centers for the conductance of electrons to the second and very simple membrane subcomplex constituted of the two anchor proteins. This membrane complex also contains a b-type cytochrome and the binding site for ubiquinone. The involvement of the b-type cytochrome with electron transport to ubiquinone is still under investigation; it is attached to both anchor proteins. The C<sub>II-3</sub> peptide has several highly conserved histidines which are candidates for linking the cytochrome to the simple membrane complex [48]. It is surprising that no more progress has been made on the analysis of this complex or the SDH enzyme by x-ray crystallography.

The reaction catalyzed by complex II is

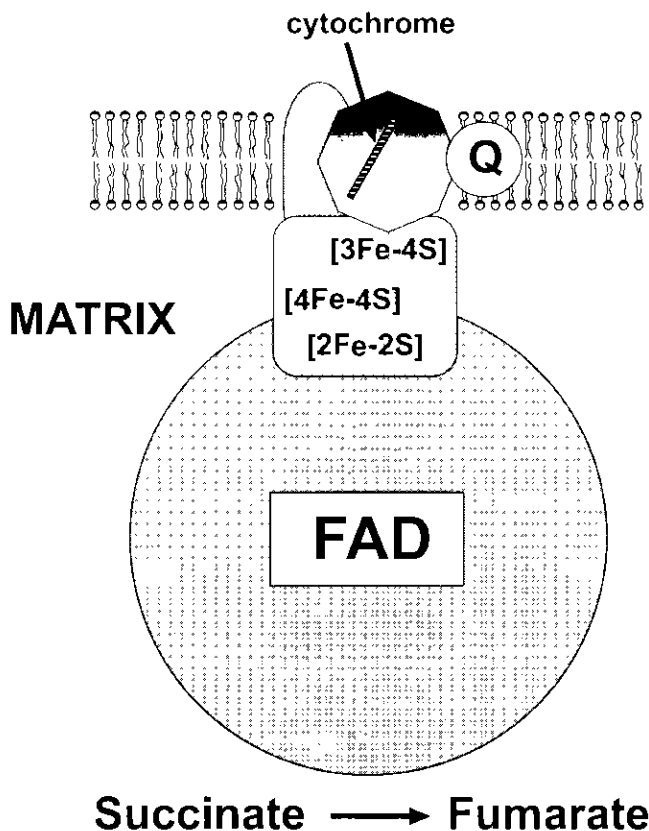


In most eukaryotic cells the reaction proceeds from left to right, not only because of the relative concentrations of substrates and products, but also because the enzyme has diode-like properties which prevent electron flow in the opposite direction [55,56]. Understanding the biophysical basis for this behavior is one of the future challenges.

Specific inhibitors continue to play an important role in biochemical studies of electron transport and the analysis of mitochondrial functions. For complex II the most useful inhibitor has been the substrate analogue malonate which binds competitively and specifically at the active site on the Fp subunit. A less common inhibitor is nitropropionic acid.

There are examples of organisms or conditions in which the reduction of fumarate becomes a physiologically relevant reaction. Facultative bacteria such as *E. coli* can proliferate under aerobic conditions and with a minimal supply of glucose when complete combustion to carbon dioxide and the most efficient exploitation of available energy resources is achieved by operating the Krebs cycle and oxidative phosphorylation. The interconversion of essential metabolites also requires the Krebs cycle. *E. coli* and other bacteria can also grow under anaerobic conditions when energy metabolism has to be altered drastically. Under such conditions the reduction of fumarate to succinate becomes an essential reaction. However, the reverse reaction is not achieved by the same SDH enzyme induced under aerobic conditions, but is instead catalyzed by a completely distinct complex named fumarate reductase, FRD. The two complexes are similar with respect to protein composition (Fp, Ip, and anchor proteins), but these peptides are encoded by two distinct operons. *SdhA*,<sup>1</sup> *sdhB*, *sdhC*, *sdhD* are represented by four cistrons on a polycistronic transcript for succinate:ubiquinone oxidoreductase, and *frdA*, *frdB*, *frdC*, *frdD* are encoded by four cistrons for fumarate:ubiquinone oxidore-

<sup>1</sup>The genes for complex II have been designated in different ways by different authors in various organisms. The designation *SDH1*, *SDH2*, *SDH3*, and *SDH4* has been adopted for yeast, while *SDHA*, *SDHB*, *SDHC*, and *SDHD* has been recommended for the mammalian genes by the Hugo/GDB Nomenclature Committee. *SdhA* - *sdhD* have been used for bacterial genes.



**Figure 5.11** Schematic representation of complex II, succinate-ubiquinone oxidoreductase. Two integral membrane proteins serve to anchor the iron-protein (Ip) and the flavoprotein (Fp) of SDH.

ductase). In *B. subtilis* there is only one anchor protein, but it is almost twice the size of the two *E. coli* peptides and may represent a gene fusion. The regulation of the expression of these operons by oxygen and glucose has been studied extensively in bacteria [40,57,58]. It remains to understand in some detail how some changes in the

primary sequence of these peptides can alter the microenvironment of the redox couples (Fe-S, FAD) to favor reactions in one direction or the other. Another noteworthy aspect of the SDH and FRD activities in bacteria is that ubiquinone serves as the electron acceptor from SDH under aerobic conditions, while reduced menaquinone serves as the electron donor via the FRD in the reduction of fumarate to succinate under anaerobic conditions. For a more detailed discussion of issues the reader is referred to the authoritative review of Ackrell and colleagues [39] in which both prokaryotic and eukaryotic enzymes are discussed.

Another interesting situation arises for eukaryotic organisms which spend part of their developmental cycle under relatively anaerobic conditions and another part of their life under aerobic conditions. For example, the sheep nematode *Haemonchus contortus* switches to a fermentative, predominantly anaerobic metabolism of the parasitic (adult) stages from the aerobic metabolism found in the free-living larvae [59]. It appears that isozymes of the iron-protein of complex II are differentially expressed in larvae and adults, and it has been argued that a switch in isozymes is responsible for the change in complex II from functioning predominantly as a fumarate reductase to the conventional succinate:ubiquinone oxidoreductase under aerobic conditions. In a related species, *Ascaris suum*, a similar switch and the existence of isozymes for the flavoprotein has been demonstrated [60]. The existence of multiple genes (isozymes) for complex II may be a general observation for some protozoa, parasitic helminths, and marine organisms such as annelids and mussels, which spend their different life cycles under different conditions [61]. It is not yet clear whether one or all of the subunits of complex II have to be changed. The problem is not only to change the direction of electron flow between succinate and fumarate, since the redox potential of all the participants must be considered. Hence ubiquinone (UQ/UQH<sub>2</sub>; Eo' = +100 mV) is generally the acceptor in succinate oxidation, while menaquinone is the donor in bacteria. Eukaryotes engaged in mitochondrial fumarate reduction must synthesize a closely related rholoquinone (RQ) [62]. Since these quinones interact with the anchor proteins, a switch in energy metabolism during development may require not only a different set of anchor proteins, but also a new pathway for the synthesis of a different quinone. Since UQ and RQ differ only in one substituent on the quinone ring (the 5-methoxy group is replaced by an amino group in RQ), the branchpoint can be postulated to occur at the end of the pathway of UQ or RQ biosynthesis. In all eukaryotes examined to date the finding of RQ has been indicative of a potential for fumarate reduction. For example, the detection of a significant quantity of RQ in the sporocysts of *Schistosoma mansoni* has been correlated with fumarate reduction as an essential pathway during the parasitic stage in the intermediate hosts (snails) where sporocysts behave as facultative anaerobes [61]. On the other hand, in *Leishmania infantum* promastigotes it had also been suggested that fumarate reduction to succinate constitutes an electron sink during anoxia. However, the absence of RQ in these organisms suggested that this reaction was absent, and studies have confirmed that these organisms produce succinate exclusively via the Krebs cycle under aerobic conditions, and go into a metabolic arrest during oxygen deprivation [63].

It has been speculated that fumarate reductase was the first activity in prokaryotes in evolution, with menaquinone as the cofactor. When oxygen concentrations were raised

in the atmosphere, an oxidative metabolism evolved which included the Krebs cycle and specifically required the conversion of an FRD activity to an SDH activity. This was achieved by gene duplication, covalent attachment of the flavin, a raise in the standard redox potentials of the iron-sulfur clusters, and the utilization of ubiquinone instead of menquinone [64]. The FRD activity was subsequently lost in eukaryotic mitochondria. However, it is conceivable that in a few facultative anaerobic eukaryotes a "reversed" evolution took place. A novel activity allowed the diversion of 5-hydroxy-6-methoxy-3-methyl-2-polyprenyl-1,4-benzoquinone to rhodoquinone instead of ubiquinone, while gene duplication and evolution yielded isopeptides which could be assembled to function as fumarate reductase again. A detailed examination of cloned genes and deduced peptide sequences will be required to resolve and support such a hypothesis.

*5.2.2.2.1 Nuclear vs Mitochondrial Location of Complex II Genes* Until recently it was correct to state that all complex II proteins were encoded by nuclear genes. This generalization has to be abandoned, because several different organisms have been found in which some of the complex II genes are found on the mitochondrial genome. In the red algae *Chondrus crispus* the *sdhB* and *sdhC* genes are found on the mt genome [65,66]; in a different red algae (*Porphyra purpurea*) and in the phylogenetically distant zooflagellate *Reclinomastix americana*, the genes for the iron-protein and the two membrane anchor peptides are found in the mitochondria [67]. These data are in agreement with the endosymbiont hypothesis for the origin of the mitochondrial genome, and they have been used to argue in favor of a monophyletic origin of mitochondria (see Chapter 2).

Eukaryotic genes for the Ip peptides (SDH2) in complex II were first cloned in 1989 [68,69], and their number has increased dramatically over the past few years. Similarly, the gene for the Fp peptide (SDH1) is available from numerous species. Cloning the SDH3 and SDH4 genes has been more challenging, because the sequence conservation across species is poor, in contrast to the Ip and Fp peptides, and probes from one organism generally do not hybridize with genes/cDNA from another distantly related organism [48]. An extensive review of the molecular genetics of complex II with an emphasis on gene cloning and regulation of gene expression has been published recently [48]. The major conclusions can be found in Chapter 4.

Gene disruption experiments in yeast have provided yeast mutants with either the Fp, Ip or anchor protein genes deleted, and such mutants can be used as recipients for novel gene constructs with specific alterations to investigate structure-function relationships [70–72]. For example, a series of chimeric Ip proteins containing yeast and human sequences were investigated to delineate a sequence between the first and second cysteine cluster, which appears to be species-specific, and when replaced by a heterologous sequence, makes it impossible to assemble complex II [73]. Until a crystal structure becomes available, such data cannot be fully interpreted.

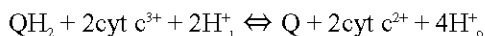
An interesting mammalian cell mutant defective in SDH activity and assembly of complex II has been described for Chinese hamster fibroblasts in tissue culture [74]. The mutant cells are respiration deficient and unable to grow in medium in which glucose is limiting. The mutation has been established to result from a single nucleotide change converting a tryptophan codon to a stop codon, thus truncating the C<sub>11-3</sub> pep-



tide before the third transmembrane segment [75]. The fate of the truncated peptide has not been determined, but the result is a complete failure of the assembly of complex II; not even an active SDH enzyme complex is formed, although the Fp and Ip peptides are made and imported normally into the mitochondria. This result can be contrasted with the findings with the *nuo21 Neurospora* mutant, where the peripheral complex is made in an active form but not attached to the membrane arm. The implications of this finding are that the Fp and Ip are not assembled separately before their attachment to the membrane. Instead, the matrix domains of the integral membrane proteins appear to participate in the folding and formation of the Fe-S centers in the Ip subunit and the simultaneous association with the Fp subunit. In this context it is worth noting that the Ip and Fp peptides cannot be separated without denaturation, and they have never been successfully reassembled *in vitro* into an active SDH enzyme.

### 5.2.2.3 Complex III

Complex III is ubiquinone-cytochrome-c oxidoreductase, and often named the bc<sub>1</sub> complex after the two cytochromes found within it; one also finds the simpler name cytochrome-c reductase in the literature. The overall reaction catalyzed by this complex is



Similar to the reaction taking place with complex I, the oxidation of one of the substrates (QH<sub>2</sub>) and the transfer of electrons to the mobile carrier (cyt c) is coupled to the transfer of protons across the inner mitochondrial membrane, and the complex therefore can be considered a proton pump. The fate of the protons will be the subject of the discussion to follow, and a more detailed mechanism for proton translocation across the membrane is also deferred for the moment.

Complex III appears to be quite similar in different species including the yeast *Saccharomyces cerevisiae*, *Neurospora*, animals, and plants. One peptide of the complex is encoded by the mitochondrial genome and incorporated as cytochrome b. The other peptides (8 in yeast, ~ 8 in *Neurospora*, 10 in mammals) are encoded by the nuclear genome, synthesized in the cytosol and imported for assembly in the inner mitochondrial membrane. Both yeast and *Neurospora* have become model systems for the study of this complex because genetic manipulations allow detailed questions to be asked about individual peptides and amino acids within each peptide, and their role in assembly, function and activity. It has been described elsewhere in this volume that in yeast even the mitochondrial gene is now subject to deliberate mutational modification (Chapter 4.5.5).

Functionally the most important subunits are the cytochromes b and c<sub>1</sub>, and the Rieske iron-sulfur protein, since they are the only ones participating in electron transfer and in the accompanying proton translocation. This fact is emphasized by the finding that the corresponding complex in the bacterial electron transport chain has only those three proteins. The role of the other proteins is difficult to deduce because they have no prosthetic groups. However, at least in *Neurospora* and potato the two large peripheral subunits appear to be processing proteases, and the largest sub-

unit I has been shown to be involved in the import and processing of proteins from the cytosol [76,77].

In the mammalian system, and to a large extent also in *Neurospora*, the elegant experimental approaches to studying complex III have primarily relied on solubilization, partial dissociation into subcomplexes, and reconstitution studies [76,78]. The original procedure by Hatefi for the isolation from mammalian mitochondria has been simplified and adapted to a smaller scale for many purposes. When too much lipid is removed during the purification, the activity of the complex declines. A most useful technique for isolating OX-PHOS complexes including complex III directly from homogenized human tissues is blue-native polyacrylamide gel electrophoresis (BN-PAGE), after solubilization of the membrane by neutral detergents [79,80]. It is suitable for the preparation of complexes in the microgram to milligram scale. As isolated from bovine and *Neurospora* mitochondria, the complex III is a dimer, a conclusion supported from electron microscopic studies of membrane crystals of the complex from *Neurospora*. It remains unclear whether dimerization is essential for the catalytic activity of the complex.

Relatively mild detergent can dissociate two subunits from the bovine complex: the Rieske iron-sulfur protein (ISP) and a small ISP-associated protein. Further dissociation with 1.5 M guanidine yields a cytochrome c<sub>1</sub> subcomplex (cytochrome c<sub>1</sub>, p9.2, p7.2), a core subcomplex, and several isolated subunits. Cytochrome b appears to remain with the core subcomplex, but a large fraction is lost in this procedure. When higher detergent concentrations are used to remove neutral lipids from the bovine complex, activity is lost, but can be restored by slow addition of phosphatidylcholine or phosphatidylethanolamine in Triton X-100. Quantitative measurements have suggested that a complex III dimer must be surrounded by a complete annulus of phospholipid. Less easily rationalized in terms of a structural model is the observation that bovine complex III has 8 to 9 tightly bound cardiolipin molecules per monomer. Their removal causes irreversible loss of activity.

All eleven mammalian complex III peptides have been purified on SDS-PAGE and used for partial sequencing and for the production of antibodies which can be used in the exploration of the topology of the complex in the inner membrane. Another experimental approach to topological studies has employed EPR techniques. An emerging model places the heme b<sub>562</sub> near the middle of the lipid bilayer, and the other heme (b<sub>566</sub>) and the Rieske iron sulfur cluster as well as cytochrome c<sub>1</sub> are on the "P-side" of the membrane (facing the intermembrane space).

Complex III, like complex I, has two reaction centers for ubiquinone, Q<sub>N</sub> and Q<sub>P</sub>. The significance of these multiple sites will become more apparent when the proton pumping mechanism will be discussed in greater detail.

Studies addressing problems related to the yeast complex III have been predominantly of a genetic nature. All eight nuclear genes for this complex have been cloned, and null mutants for each gene have been isolated. With the exception of the mutant affecting the acidic subunit VI these mutants are respiration-deficient. Therefore, even the subunits without prosthetic groups are necessary for an active complex III, and one can speculate that they are required for assembly and stabilization of the complex. Among the many *pet* mutants analyzed [81], mutants with a defective

ubiquinone-cytochrome-c oxidoreductase activity can be found, although a direct selection or enrichment for such a specific defect is not possible. Thus, *pet* mutants having verified nuclear mutations must be further screened by biochemical assays to eliminate mutants affecting mitochondrial protein synthesis or the ubiquinone biosynthetic pathway [82]. Among the mutants with a specific lesion in complex III activity several classes have been distinguished:

1. Mutants with alterations in the eight nuclear genes for the protein subunits of the complex. As discussed above, null mutants are respiration-deficient. In many of these the cytochrome b is also absent, presumably because it needs to be stabilized by association with other subunits.
2. A number of mutants are unable to make a cytochrome b detectable by spectroscopy, even though all structural genes in the nucleus and the COB gene on the mtDNA are normal. It has been discussed in another chapter (Chapter 4) on the expression of mitochondrial genes in yeast that a variety of imported proteins are required for cytochrome b mRNA maturation (group I intron splicing and processing of the 5' terminal), and for mRNA stabilization. The COB gene encodes a protein which appears to function both in 5' end processing and mRNA stabilization (translational initiation?). Additional nuclear gene products are necessary for efficient translation of the COB mRNA, which in turn also affects the splicing of the intron, because the COB intron encodes a maturase (see Chapter 4). Another consequence may be a defect in cytochrome oxidase (complex 4) as a result of defective splicing of the COX1 mRNA.
3. Nuclear gene products are also required for the addition of the prosthetic groups to the various subunits. Hemes have to be covalently attached to apocytochromes, and another gene product, Bcs1p, has been implicated in the "maturation" of the Rieske protein. There is in general still some uncertainty about whether iron-sulfur centers assemble spontaneously, and hence the precise function of the BCS1 gene product is not known.
4. Another set of proteins defined by nuclear mutations are involved in the "late stages of the assembly pathway" [82]. This is a problem which so far has been defined exclusively by a genetic approach in yeast. Similar assembly factors have been identified for complexes IV and V. The mechanisms and pathways of assembly of the complexes in the ETC represent a major frontier in the quest for understanding mitochondrial biogenesis.

Cytochrome-c reductase (complex III) from potato mitochondria has recently received scrutiny, as attention has been attracted more broadly to plant mitochondria. Similarities to the complex in mammals and yeast may not be surprising, but when sequencing information became available for comparative studies, the so-called core proteins from the potato complex showed the highest sequence identity with the mitochondrial processing peptidase (MPP) from mammals and yeast [77]. These enzymes are involved in the cleavage of matrix targeting sequencing from precursors

imported from the cytosol. It was subsequently verified that a bifunctional protein exists in potato mitochondria (all plants? ) which plays a role in electron transport as well as in protein import. The complex is therefore referred to as the cytochrome-c reductase/processing peptidase complex (see Chapter 4.6). Its overall composition (at least 10 subunits) resembles the mammalian and fungal complexes.

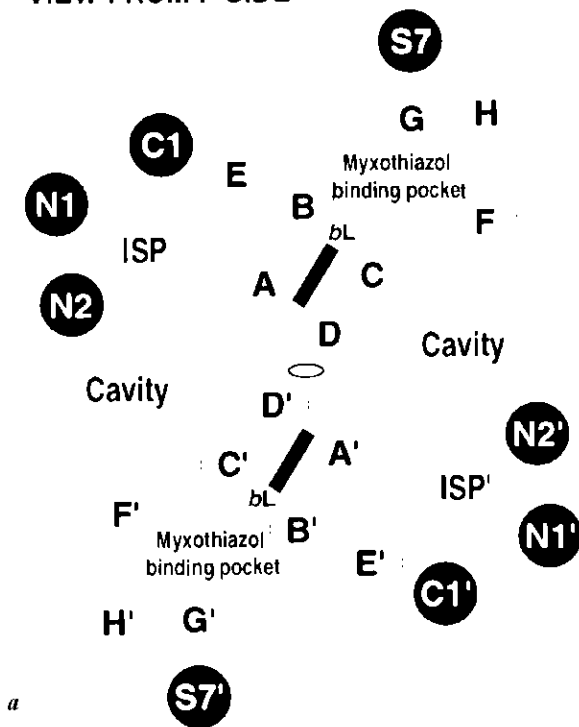
A crowning achievement in the study of complex III was the recent completion of the complete crystal structure of the complex at 2.9 Å resolution by the groups of Yu, Deisenhofer, and colleagues [83]. Following on the heels of the determination of the structure of complex IV (see below) it represents another major landmark in the understanding of the complexes of the mitochondrial electron transport chain. An atomic model has now become available to relate the proposed reaction mechanisms of QH<sub>2</sub> oxidation and the reduction of cytochrome c to the architecture and topology of the complex.

The model confirms, refines and expands the structural features deduced from biochemical studies. In the crystal two bc<sub>1</sub> monomers interact to form a dimer with a twofold axis of symmetry perpendicular to the membrane. Each monomer has 13 transmembrane helices: eight are from cytochrome b, one is from cytochrome c<sub>1</sub>, one from the iron sulfur protein, one from subunit 7, and one is still unassigned (Figure 5.12). On one side of the membrane more than half of the molecular mass of the complex extends 75 Å into the matrix. It consists mainly of the so-called core proteins which have a structural rather than a functional role. The peripheral core 2 proteins contribute significantly to the stabilization of the dimer, in addition to the interaction of the cytochrome b helices within the membrane. The two core proteins of the mammalian bc<sub>1</sub> complex have homology to the  $\alpha$  and  $\beta$  subunits of the mitochondrial matrix protein processing peptidase (MPP), and it is suggested that the soluble mammalian MPP has a similar structure. As discussed above, in plants the MPP is an integral part of complex III. A detailed side view of the complex is shown in Figure 5.13.

On the outside of the membrane (P side) one finds the Rieske iron sulfur cluster and the domain of cytochrome c<sub>1</sub> containing the heme. The electron density for this latter region is relatively poor, and the authors ascribe this problem to mobility in the crystal, which may in fact reflect a mobility required for interactions with, and electron transfer to, cytochrome c. Two additional heme irons could be located within the membrane domain, corresponding to heme b<sub>L</sub> and heme b<sub>H</sub>, respectively. The identification and distinction between the two hemes of cyt b was also aided and confirmed by the availability of difference density maps comparing the native crystals with crystals obtained with the inhibitors antimycin A or myxothiazol. The different targets of these inhibitors had been assigned from biochemical and spectroscopic studies (see below).

Of special interest are the distances between the heme irons in b<sub>H</sub>, b<sub>L</sub>, and c<sub>1</sub> and the [2Fe-2S] cluster, in part to explain and confirm previous spectroscopic studies, and understand the rate of electron transfer. In the dimer the distances between two b<sub>L</sub>'s and two b<sub>H</sub>'s are comparable to the b<sub>H</sub>-b<sub>L</sub> distance within a monomer, raising the possibility of electron transfer between the monomers.

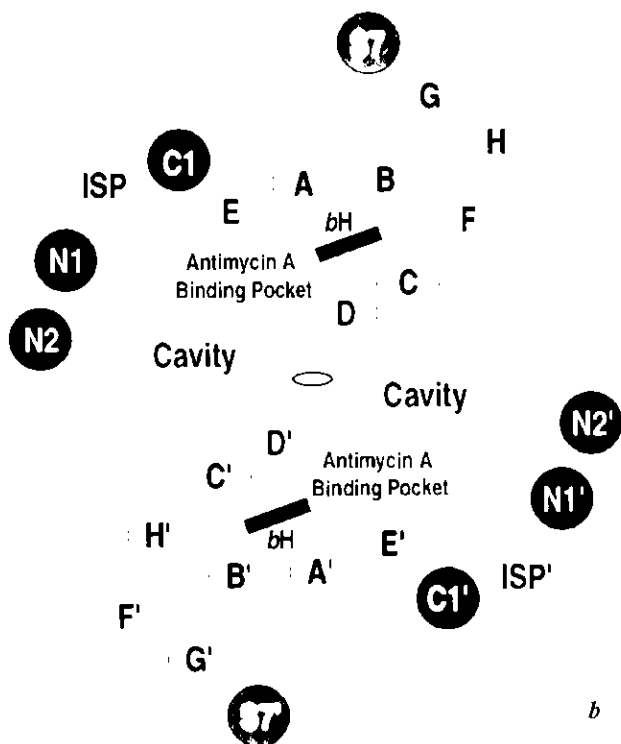
## VIEW FROM P SIDE



The overall model with its "cavities" and "pockets" clearly invites further speculations about the accessibility of the complex to ubiquinone, antimycin and myxothiazol, and ultimately about the translocation of protons and the operation of the Q cycle.

Specific inhibitors of electron transport have been valuable as a means of arresting electron flux at known locations for the purpose of spectroscopic studies, for example, and to "isolate" a portion of the chain for detailed study. They have also been useful and revealing probes of structure, and in some cases they have been useful to arrest electron flow within a complex to establish upstream and downstream portions of the complex. In the case of complex III, the antibiotic antimycin A is the best known of the inhibitors, but a variety of natural and synthetic inhibitors have been characterized. Ini-

## VIEW FROM N SIDE



**Figure 5.12** A and B: Schematic representation of the transmembrane helices in the dimeric complex III seen from the P and N (matrix side). Since some of the helices are tilted or have kinks, the two patterns do not coincide precisely.

tially their utility was in the definition of a specific block in the electron transport chain downstream from complexes I and II and upstream from cytochrome *c*, and in the localization of the “coupling sites” for oxidative phosphorylation. Later they were exploited in defining the distinction between center  $Q_N$  or center  $Q_P$  and in elucidating the protonmotive *Q* cycle (Figure 5.14).

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*a*

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*b*

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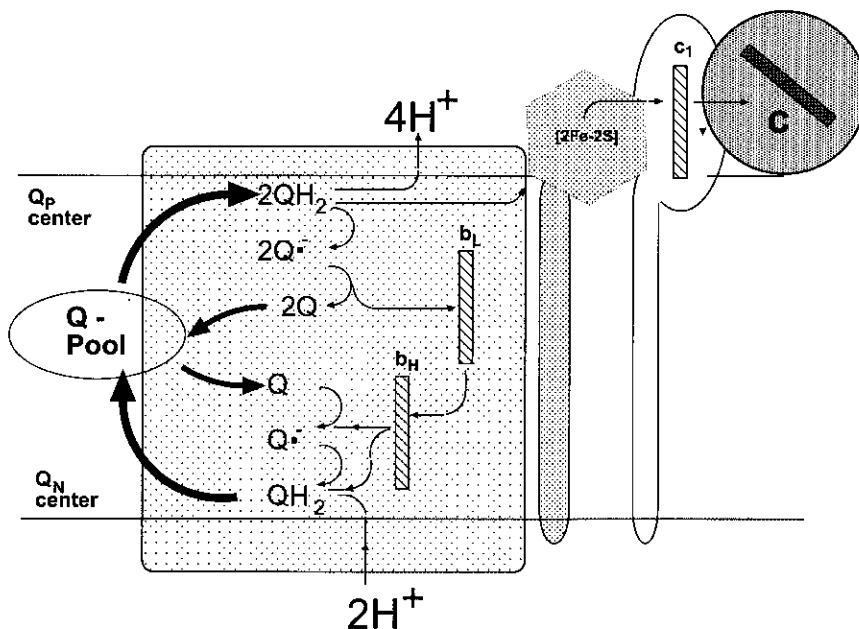
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*d*

**Figure 5.13** **A:** Side view of complex III. The matrix side is at the bottom. Cytochrome  $c_1$  extends into the intermembrane space. The membrane-spanning helices of the various subunits are in the region indicated by the middle arrow (42A). **B:** Stereoview of the transmembrane helices of a monomeric complex III. The large domains extending into the matrix are omitted (bottom). **C:** Stereoview from the outside showing the myxothiazol binding pocket. **D:** Stereoview from the matrix side showing the antimycin A binding pocket. (From Reference 83 with permission. The figure was generously provided by Dr. Yu.) (See color plates.)

The formulation of the Q cycle [84–87] required the postulate of two quinone reaction sites (see below). The  $Q_N$  or quinone reduction center is located on the matrix side (N-side) of the inner membrane and is associated with the recycling of half of the electrons back into the quinone pool and the uptake of protons from the matrix. It is inhibited by antimycin A, funiculosin, and hydroquinoline-N-oxides, inhibitors which interfere with the electron transfer from heme  $b_H$  to Q or  $QH^-$ . At the  $Q_p$  center electrons from reduced ubiquinone are accepted and divided into two pathways: half for recycling, and half for transfer via the iron-sulfur center and cyt  $c_1$  to cytochrome c. It is located near the outer face of the inner membrane, and protons are released into the



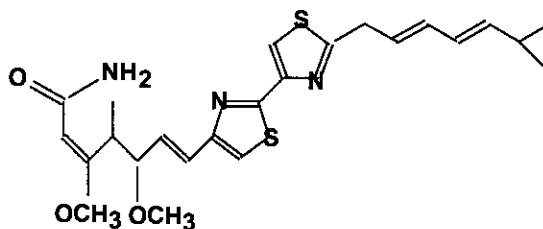


**Figure 5.14** The Q-cycle and electron flow through complex III.  $\text{QH}_2$  is oxidized, and electrons are transferred to the mobile carrier cytochrome c (upper right in the figure). For a detailed explanation see the text. (Adapted from References 87 and 141).

intermembrane space. A number of different compounds can inhibit at the  $\text{Q}_\text{p}$  center: 2-hydroxy-1,4-benzoquinone derivatives, stigmatellins, and MOA inhibitors containing the E-b-methoxyacrylate group (MOA-stilbene, myxothiazol).

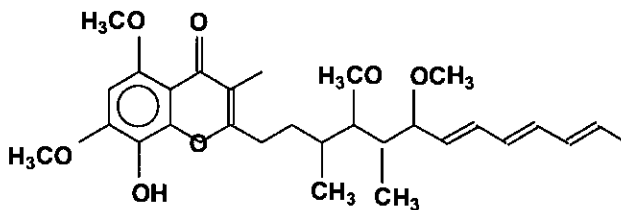
A general review of the approaches and methodologies used with these inhibitors (Figure 5.15) has been written by Link and colleagues [88]. Having established the

Myxothiazol

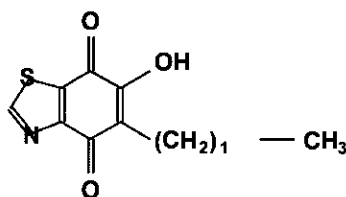


Stigmatellin A

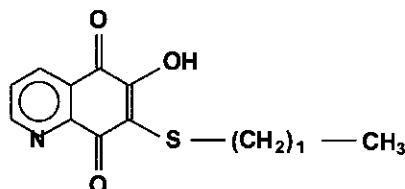
*a*



undecylhydroxydioxobenzothiazole  
UHDBT

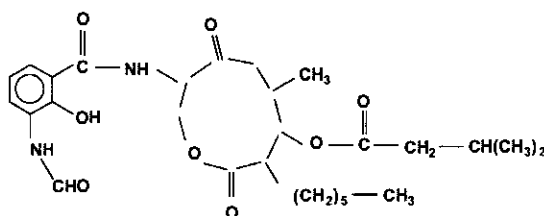


heptadecylmercaptohydroxyquinoline quinone  
HMHQQ

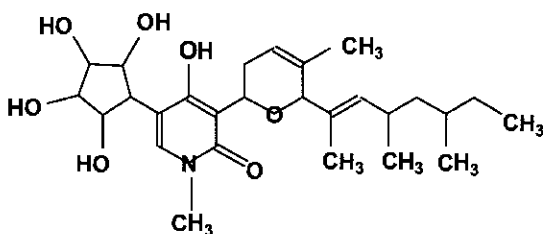


*b*

Antimycin A



Fusiculosin



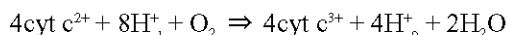
*c*

**Figure 5.15** Different subclasses of inhibitors frequently used in the study of complex III. Inhibitors in groups A and B inhibit at the  $Q_p$  center, while inhibitors in group C inhibit at the  $Q_N$  center on the matrix side of the inner membrane.

specificity and efficacy of each inhibitor in arresting electron transport, additional studies have included light and EPR spectroscopy, the isolation of resistant mutants of a variety of species, the identification of mutated amino acid side chains in specific peptides from such mutants, characterization of mutations induced by site-directed mutagenesis [89], and the synthesis of additional analogues, for example, antimycin A analogues [90].

#### 5.2.2.4 Complex IV

Complex IV is generally referred to as cytochrome-c oxidase. The overall reaction catalyzed is the following:



Molecular oxygen is the terminal electron acceptor, the mobile carrier cyt c is reoxidized, and four protons are pumped from the matrix to the intermembrane space.

After three decades of study and analysis this complex has the distinction of being the first complex of the ETC to have its high resolution crystal structure determined at  $\sim 2.8$  Å resolution. Two landmark papers on the bovine complex IV have presented the largest and most complex membrane protein to be solved at this resolution to date [91,92]. A monumental achievement, they mark a major milestone in the history of electron transport and respiration. As always, these papers settled some older arguments and answered a number of questions quite definitively, but a wealth of new questions can now be raised to understand at the atomic level how electron transport is intimately coupled to proton pumping. Only high resolution computer images with the added capability of rotating the image and zooming in and out of the structure can give a true sense of this achievement and its powerful influence on future thinking about these fundamental processes in bioenergetics. As one review summarized the current situation: "The monster is subdued, but far from tamed" [93].

The functionally homologous but simpler bacterial structure also became available in 1995 [94], by itself a major achievement. A remarkable conservation of structural features helps to emphasize those aspects most basic and essential for function. Certain differences will emphasize the requirement for regulatory mechanisms in higher organisms which may not exist or may be different in bacteria.

A discussion must start with the composition of the complex. The mammalian complex contains 13 subunits, the yeast complex is composed of 9 subunits, and one may expect to see further variations in the total number of subunits in different organisms. In all organisms the three largest subunits (I, II, III) are encoded by the mitochondrial genome and synthesized in the matrix. For the remaining subunits the nomenclature can be confusing to the uninitiated: in the mammalian complex we have subunits IV, Va, Vb, VIa, VIb, VIc, VIIa, VIIb, VIIc, and VIII; in yeast the subunits are IV, V, VI, VII, VIIa, VIII. And while a biochemist may refer to subunit VIIa in yeast, a geneticist might refer to it as the COX9 gene product.

A large number of biochemical and elegant spectroscopic analyses had established some time ago that the complex contained two hemes ( $aa_3$ ), and two copper centers. In fact, the cytochromes a and  $a_3$  were among the first described by D. Keilin in 1925. The x-ray structure has probably been most definitive in defining two additional metal centers containing Mg and Zn. Studies on the simpler bacterial cytochrome c oxidases and comparisons of homologous peptides have given clues about the association of these important metal centers with specific peptides which are now totally confirmed for yeast and mammals. Subunit I binds the heme a and heme  $a_3$  prosthetic groups and also forms the  $\text{Cu}_b$  redox center. Subunit II binds the  $\text{Cu}_A$  center. Subunit III is likely to be intimately involved in proton pumping and in modulating the elec-

tron transport through the metal centers, and thus, subunits I–III form the functional core of the enzyme. The other subunits may perform regulatory functions, or play a role in insulation, stabilization, or assembly. Such a regulatory function is indicated by the existence of tissue-specific isoforms for the subunits VIa, VIIa, and VIII in mammals [95,96].

In by now standard fashion, the yeast nuclear genes have been disrupted one at a time, and in the absence of either subunit IV, VI, VII, or VIIa there is no cytochrome-c oxidase activity and cytochromes aa<sub>3</sub> are missing. A knockout of the COX8 gene (subunit VIII) causes only a 20% reduction of activity at normal aa<sub>3</sub> levels. There are two isoforms of subunit V, either one is required for activity, but complexes with either Va or Vb differ slightly in their kinetic and spectroscopic properties. The yeast complex may have additional subunits, making it more similar to the mammalian complex, but it has to be clarified whether they are bona fide subunits, scaffolding proteins required only for assembly, or simply contaminants.

For many years laboratories have attempted to define the topology of these subunits within the inner membrane by reactions with membrane impermeable reagents, by cross linking and coprecipitation, and by an exploration of the location of epitopes with a large variety of monoclonal antibodies [97,98]. In light of the solution of the crystal structure these studies need not be reviewed here, but the development of the technology and reagents will continue to be useful tools in the examination of the enzyme, particularly in comparisons between the normal enzyme and enzymes derived from individuals with mitochondrial mutations in the genes for the three largest subunits [98].

There are 28 transmembrane helices contributed by the various subunits. Viewed from the top of the membrane, they form an irregular cluster surrounding the metal centers, in effect solubilizing the metal sites within the lipid bilayer, and also “insulating” the electron path from the surrounding lipid layers. Many helices at the outside of the cluster probably have no other function than to serve in the assembly and stabilization of the complex. On the other hand, most of the transmembrane helices are not parallel to each other, and they are not vertical to the plane of the membrane. Three subunits (Va, Vb, and VIb) are not within the membrane, and are associated as peripheral membrane proteins with extramembrane domains of the membrane subunits.

In considering the structure in detail, it is profitable to decompose the problem into separate structure/function relationships. The central issue is the path of electrons from the substrate ferrocycytochrome c to oxygen. Another problem of fundamental importance is to understand the proton pumping activity associated with electron transport. Finally, regulatory functions, stabilization of the complex, and the assembly mechanism may be addressed from a consideration of the high resolution structure. The emergence of the structure in two major stages reflects this order of priority. The representation of the metal sites within the overall contours of the complex and in relationship to the membrane was the first major achievement [91], followed one year later by the entire structure [92]. The solution of the x-ray structure of this complex (Figures 5.16, 5.17) was a monumental achievement and a milestone in the history of bioenergetics. Highly detailed graphic representations of the structure can now be found at numerous sites on the internet.

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**Figure 5.16** X-ray structure of complex IV. **A:** Side view showing the transmembrane segments of the various subunits; matrix side at the bottom. **B:** View from the cytosolic side. (The photographs were kindly provided by Dr. S. Yoshikawa of the Himeji Institute of Technology, Hyogo, Japan.) (From Reference 92, with permission.) (See color plates.)

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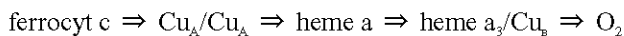
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**Figure 5.17** X-ray structure of complex IV. Stereo images of the  $C_{\alpha}$  backbone tracings. **A:** Subunits I, II, and III. **B:** Subunits IV, Va, Vb, VIa, VIb, VIc, VIIa, VIIb, and VIII. (The photographs were kindly provided by Dr. S. Yoshikawa of the Himeji Institute of Technology, Hyogo, Japan.) (From Reference 92, with permission.) (See color plates.)

The two metal ions  $\text{Mg}^{2+}$  and  $\text{Zn}^{2+}$  are not redox centers. The  $\text{Zn}^{2+}$  ion is quite some distance away from the "action," and its major function appears to be in the stabilization of the three-dimensional structure of the complex. The  $\text{Mg}^{2+}$  ion on the other hand is between  $\text{Cu}_A$  and heme  $a_3$ , and, although not directly involved, it may have more than a simple stabilizing role, either in electron transport (and its direction), or in the coupling with proton transfer.

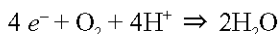
While the spatial arrangements and precise distances between the other metal centers were finally determined by the crystal structure, numerous ingenious spectroscopic studies had contributed to a path for the electrons which in a simplified form has the following appearance:



In this scheme the  $\text{Cu}_A$  center (with two copper ions) is located near the surface of the complex facing the intermembrane space and hence it is able to be brought in close contact with the reduced cytochrome  $c$ . The two heme  $a$  groups are buried within the complex as electron carriers with a redox potential determined by the surrounding protein. The unique feature is the bimetallic heme iron-copper reaction center which is also the site to which oxygen approaches for its activation and conversion to water. To understand more detail, one must understand the nature and redox behavior of the  $\text{Cu}_A/\text{Cu}_A$  center, and most importantly, the mechanism of the transfer of electrons to molecular oxygen from the heme  $a_3/\text{Cu}_B$  combination.

The  $\text{Cu}_A/\text{Cu}_A$  center has the  $[2\text{Cu}-2\text{S}_\gamma]$  cyclic structure in which the two copper ions are coordinated with two cysteines, two histidines, a methionine and a peptide carbonyl of a glutamate of subunit II. These ligands, except for the carbonyl group, had been proposed previously on the basis of mutagenesis experiments which were fully confirmed by the x-ray structures. The presence of two copper ions per site A had long been uncertain and controversial. An insightful account of the history of the  $\text{Cu}_A$  site in cytochrome oxidase has recently been written by H. Beinert [99]. It is highly instructive in tracing the development of ideas about Cu in complex IV, in emphasizing the development of methodology and techniques (purification, spectroscopy, molecular genetics). Many general concepts in protein structure with special emphasis on metal-protein (ligand) interactions ultimately had to be applied to the understanding of a complex with seven metal ions, three of them Cu. It now appears that there are major similarities to the  $[2\text{Fe}-2\text{S}]$  centers, where one-electron transfers can be facilitated by delocalizing the charge between the two metal ions.

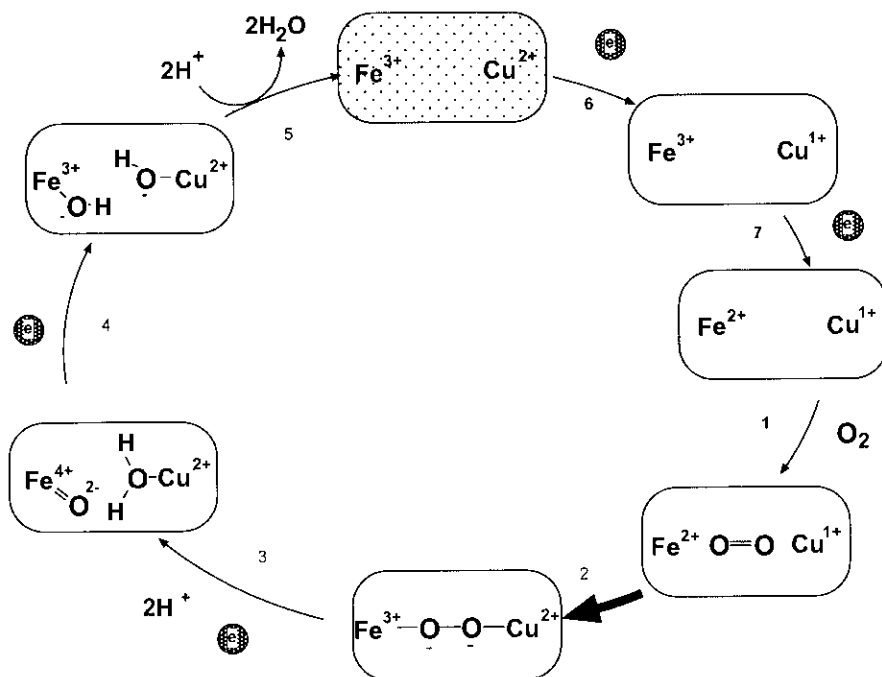
The most unusual and interesting structure is the binuclear heme  $a_3$  -  $\text{Cu}_B$  center, and major interest in this center arises from the nature of the reaction occurring there. It is usually presented as a simple addition of electrons and protons to oxygen:



However, details of this process are more complicated [100]. With 20% oxygen in the atmosphere, the organic material on the earth's surface would spontaneously burst into flame if the activation of molecular oxygen were simpler and energetically more

favorable. The low normal reactivity of  $O_2$  arises from the triplet electronic ground state of oxygen, with two unpaired electrons, which makes both single and two-electron transfers from singlet state donors kinetically slow. Thus, the thermodynamically highly favorable reduction of oxygen to water can be kept in check, except at high temperatures, or at reaction centers such as the one under discussion. The scheme of oxygen activation proposed by Babcock and Wikstrom [100] is shown in the following schematic diagram (Figure 5.18).

The cycle starts with the addition of two electrons from cytc to form  $Fe(II)/Cu(I)$ . Oxygen binds to the heme iron, but the proximity of the copper ion allows two electrons from within the center to be transferred to oxygen, converting it to the peroxy ion ( $O_2^{2-}$ ), and resulting in the oxidation of the metal ions to  $Fe(III)/Cu(II)$ . The first of the four electrons from the upstream heme a enters, reducing the copper to the  $Cu(I)$  form again, and allowing the oxygen to accept the first proton. Electrons are again redistributed between the Fe and Cu within the center. Upon addition of a second proton the oxygen is split, forming the ferryl species of heme  $a_3$  and water coordinated to the cupric ion. The second electron reduces the  $Cu(II)$ , and proton redistribution leaves  $Fe(III)$  and  $Cu(II)$  each coordinated with a hydroxyl ion. The hy-



**Figure 5.18** Proposed sequence of reactions for transferring four electrons from cytochrome c to an oxygen molecule to form two molecules of water. Electrons are transferred in steps 1 and 2 before oxygen becomes associated with the two metal ion centers, Fe and Cu. More electrons and protons are added (steps 5 and 6) until water molecules are released. (Adapted from Reference 101.)



droxyl ions are released as water following the addition of two more protons, and two more electrons are added stepwise to return to the starting position Fe(II)/Cu(I). Many of these steps have been resolved by optical measurements at low temperatures, or by time-resolved Raman spectroscopy. For the more intricate biophysical, thermodynamic and quantum mechanical aspects of this problem the reader is referred to the specialized literature (e.g., Reference 100 and references therein).

A most important consequence arises when this overall process is perturbed by the premature departure of oxygen. Partial reduction of oxygen yields the superoxide radical ( $O_2^-$ ), from which hydrogen peroxide ( $H_2O_2$ ) or the hydroxyl radical (OH) can be derived. These species are often referred to collectively as the "reactive oxygen species," or ROS, and their production is strongly implicated in oxygen toxicity and mutagenesis of mitochondrial DNA (see Chapter 7). A more elaborate discussion of the ROS and their reactions will be presented in section 5.7 below. In the present context it is important to clarify whether reactive oxygen species produced in mitochondria arise primarily from incomplete reduction of oxygen in complex IV. It has been estimated that a significant fraction (a few percent ??) of oxygen entering mitochondria is reduced only partially, and this fraction may increase under abnormal conditions or as a result of electron transport complexes impaired by mutations. Normal individuals suffer from various afflictions when exposed to greater than 21% oxygen, and prolonged exposure can lead to lung damage. Premature babies exposed to higher than normal oxygen concentrations in incubators can develop retrolental fibroplasia (ocular damage) unless carefully monitored and given  $\alpha$ -tocopherol as a free radical scavenger. It is likely that mitochondrial ROS production occurs not at the site designed for reduction of oxygen, but rather from the reaction of the highly soluble and diffusible oxygen with reduced upstream sites of the ETC. In other words, a leakage of electrons can occur from high potential sites, especially in damaged or abnormal mitochondria.

The complete solution of the structure of complex IV in mammals and of the homologous simpler complex in bacteria has greatly stimulated the discussion of the mechanism of proton pumping coupled to electron transport [92,94], but here even a structure at 2.8 Å resolution cannot give a definitive answer. Previous approaches employing mutagenesis had suggested the existence of two proton channels: one for protons which become associated with oxygen to form water, and another for the protons pumped across the membrane. From theoretical considerations one would expect the proton channels to consist of a network of hydrogen-bonded side chains whose  $pK_a$ 's would be controlled by conformational changes associated with redox reactions. Acidic groups on either side of the membrane must be alternately accessible, and their  $pK$  must vary depending on the oxidation state of the enzyme. Protons are shuttled through the protein complex along a string of residues that have been referred to as a "proton wire" [see Reference 103 for a recent discussion]. Several such networks were found, and included in these localized structures were cavities likely to contain water molecules which can participate in proton conduction. Since the original x-ray structures were reported in 1995, additional structures have now been examined of the enzyme in the fully reduced, fully oxidized, azide-bound, and carbon monoxide-bound states [102]. These studies greatly refine the conformational changes associ-

ated with the redox reaction and contribute to the formulation of more specific proton pathways and models. Nevertheless, these postulated pathways remain only candidates for the time being [103]. Their identification may suggest additional mutagenesis experiments. They also will become significant in comparisons between phylogenetically distant species, since functionally important side chains are likely to be conserved.

Finally, one must also identify a water channel for the escape of the product,  $H_2O$ , and possibly a pathway through which the substrate  $O_2$  can approach the heme  $a_3/Cu_B$  reaction site. Potential pathways have been suggested from a consideration of the crystal structure, but none of them are wide enough in the static crystal structure to accommodate these molecules. Thus, rapid, reversible conformational changes have been postulated to operate in the opening and closing of such channels. Crystal structures of the complex in other oxidation states will be required to resolve these outstanding issues.

In closing this section one should not dwell on the outstanding issues and unsolved problems. Instead it should be recognized that since the discovery of the *Atmungsferment* by O. Warburg almost seven decades ago, this enzyme has been one of the most important objects of study in the area of bioenergetics. As such it has attracted the attention of some of the greatest minds in the history of biochemistry. The whole structure at 2.8 Å resolution now represents a crowning achievement. Words fail to describe it fully, but most institutions and many individual laboratories can now obtain the structure via the internet, or on computer disks for display on a workstation. When combined with three dimensional vision achieved with special goggles, the inspection and exploration of this structure becomes an awesome spectacle even for the nonspecialist.

### 5.3 ELECTRON TRANSPORT IN OTHER ORGANISMS

The description provided above for the components of mitochondrial electron transport (there is also electron transport in chloroplasts) appears to be almost universally applicable to mitochondria in animals, plants, and most of the other species of eukaryotic organisms. However, this should not lull one into thinking that once one understands one mitochondrion, one understands them all. Yeast (*S. cerevisiae*) mitochondria lack a complex I of the kind described above. An alternate pathway for the oxidation of NADH in yeast will be presented below. Plant mitochondria have several additional capabilities which make them unique [104–106].

#### 5.3.1 NAD(P)H Dehydrogenases

The first distinctive difference between plant and animal mitochondria is in the oxidation of NADH [107–110]. Complex I in either system can accept NADH as substrate when it is produced in the matrix from reactions of the Krebs cycle, for example. NADH produced in the cytosol has to be transferred into the matrix by a shuttle system in animal mitochondria (see Chapter 6), but plant mitochondria have

an external NADH dehydrogenase that can oxidize NADH and transfer reducing equivalents directly to ubiquinone or complex III. The reaction is insensitive to rotenone and generates ATP with a P/O ratio of 2 or less. A separate dehydrogenase dependent on  $\text{Ca}^{2+}$  on the outer surface of the inner membrane is specific for NADPH [107]. No shuttles are required to deal with cytoplasmic sources of NADH (from glycolysis) or of NADPH (controlling the pentose shunt). These two activities are differentially sensitive to diphenyleneiodonium (DPI), with NADPH oxidation being irreversibly inhibited at submicromolar concentrations [111]. The two enzymes appear to be single, relatively small polypeptides (32, 55 kDa) [107]. A third rotenone-insensitive, nonphosphorylating NADH dehydrogenase made up of two 43-kDa subunits has been localized on the matrix side of the inner membrane [112].

The full physiological implications of the existence of these external and internal nonphosphorylating NADH dehydrogenases are not yet understood. Loosely speaking, these enzymes may participate in the efficient interconversion of a variety of intermediates required for rapid plant growth. The most puzzling aspect is the presence of two internal NADH dehydrogenases, one of which is very similar to complex I in animal mitochondria. Do both of these enzymes select substrates from a common pool, and what controls the relative affinity of NADH for one or the other enzyme? The rotenone-insensitive dehydrogenase appears to have a low affinity and thus may operate under certain conditions when NADH levels build up from a highly active Krebs cycle. It has been suggested that it is exclusively linked to the cyanide-resistant electron pathway (see below), providing a totally nonphosphorylating pathway for endogenous NADH oxidation [106].

### 5.3.2 A Cyanide-Insensitive Electron Pathway

Another, initially very puzzling finding was the observation of sometimes significant residual respiration by plant mitochondria in the presence of CO, azide, or cyanide [105,113]. Electrons from ubiquinol appear to have two choices, which may be regulated by physiological conditions or depend on the tissue type or developmental stage: they can either enter the conventional pathway via complex III and cytochrome oxidase to oxygen, or they can reach oxygen via a specific oxidase unaffected by the classical inhibitors. The characterization of the alternate oxidase was made more difficult because it had no characteristic absorption spectrum and did not exhibit electron paramagnetic resonance signals. The enzyme also appeared to be extremely labile, and not surprisingly it was not a soluble enzyme. The cloning of a cDNA encoding this protein, originally from the voodoo lily, *Sauromatum guttatum* [114], and subsequently from other species, has greatly facilitated the study of this enigmatic terminal oxidase. By expressing the voodoo oxidase cDNA in the yeast *S. pombe* it could be demonstrated quite conclusively that a single polypeptide (~32 kDa) was sufficient for introducing alternate oxidase activity into the *S. pombe* mitochondria [115]. It was cyanide and antimycin resistant. The protein is an integral membrane protein with two transmembrane segments. The N-terminal and C-terminal domains are exposed to the mitochondrial matrix. From sequence comparisons highly conserved motifs have been found in the C-terminal domain which make it likely that the

active site is constituted of a coupled binuclear iron center similar to that found in ribonucleotide reductase or methane monooxygenase [see Reference 115 for recent references]. The expected ligands for the irons are two histidines, two glutamates, one aspartate, and two water molecules. The enzyme is active in the reduced form; changing the redox status of the sulhydryl disulfide linkage affected the electron flux in the transgenic yeast mitochondria. The functional expression of this enzyme in yeast mitochondria in combination with site-directed mutagenesis will help to answer numerous questions about structure-function relationships in this enzyme.

Electron flow through the alternate pathway does not lead to ATP formation. In other words, there is no proton translocation across the inner membrane associated with this pathway. In some special plant tissues the pathway serves in thermogenesis (see Section 5.6.3), but like rotenone-insensitive NADH dehydrogenase in the matrix, the physiological significance of this pathway is still somewhat obscure. It has been considered to act as an overflow to drain off the energy of carbohydrates when they are in excess of demand [106]. For example, the interconversion of intermediates via the Krebs cycle could continue without constraints by a membrane potential or ADP availability (see below). Since the contribution of this pathway depends on the tissue, and is developmentally regulated, for example in fruit ripening and storage organs [105], much attention has been devoted to understanding the regulation of its activity. Transcriptional regulation of gene expression is an obvious focus of attention, now that the gene has been cloned. However, posttranscriptional, biochemical regulatory mechanisms are also under consideration. In the voodoo lilly (*Sauromatum*) an inducer of heat production and cyanide-resistant respiration called "calorigen" had been known for over 50 years, and it was recently identified as salicylic acid [116]. There is speculation that salicylic acid is a transcriptional inducer, but posttranslational modifications of the protein may also be required [114].

### 5.3.3 NADH Oxidation in Yeasts

Budding yeast, *Saccharomyces cerevisiae*, the model system for almost everything a cell biologist wishes to study, does not have a complex I of the type described for animals. This became generally apparent from the absence of sensitivity to rotenone or piericidin, and the lack of site I phosphorylation, although under some conditions the coupling site I may be induced (see below). Later, *S. cerevisiae* mtDNA proved the exception by having no genes encoding subunits for complex I which had been recognized in most other species. When NADH oxidation was measured with yeast mitochondria, both internal and external NADH dehydrogenase activities could be detected, that is, one enzyme had an active site facing the intermembrane space, while the other used NADH generated in the matrix. NADH itself cannot pass through the inner mitochondrial membrane.

The NADH dehydrogenases found in *S. cerevisiae* and also in *S. pombe* are likely to be related to the rotenone-resistant enzymes found in plants and bacteria. Some of these enzymes and their genes have been characterized [117–119]. They contain FAD and reduce ubiquinone ( $Q_6$ ) as well as a number of related and unrelated electron acceptors. A single polypeptide with apparent molecular mass 53 kDa believed to be the

external enzyme was found to be induced about 5- to 10-fold in the absence of glucose. The internal enzyme is a single 57.2 kDa peptide [119]. This yeast enzyme can be expressed as an active enzyme in *E. coli* [120], and most strikingly, it has been expressed from a mammalian expression vector in a complex I-deficient Chinese hamster cell mutant [121]. It is localized in the hamster mitochondria and restores the capacity for respiration and oxidative phosphorylation in these cells. In other words, as a single peptide it functionally complements the entire complex I consisting of approximately 43 peptides.

Generalizations about “yeasts” should be taken with a grain of salt; there are many different species of yeasts, and some of them do appear to have a “conventional” complex I. The identification is based on several criteria. The detection of rotenone-sensitivity is suggestive; the isolation of large complexes (~700 kDa) is more convincing. Finally, sequencing the mitochondrial DNAs from several of these species has revealed the presence of ORFs with obvious homology to the mitochondrial genes for complex I (ND1, ND2, ..., ND6, ...) found in many organisms. A subdivision of yeast species into strictly aerobic yeasts and facultatively fermenting yeasts may relate to the capacity for complex I formation. The presence of a complex I-like activity has so far been deduced for the yeasts *Candida pinus*, *Cryptococcus albidus*, *Rhodotorula minuta*, *Trichosporon beigelii*, *Candida parapsilosis*, *Pichia guilliermondii*, *Clavispora lusitaniae*, *Hansenula polymorpha*, and others [122,123].

### 5.3.4 Energy Metabolism and NADH Oxidation in Trypanosomes

The unusual organization and expression of the mitochondrial genome in a group of protozoa with kinetoplasts has already been discussed in a previous chapter. There are two major taxonomic groups: the trypanosomatids (e.g., *T. cruzi*, *T. brucei*, *L. tarentolae*), and the cryptobiids (e.g. *T. borreli*). These parasites have a complex life cycle which includes stages in a vertebrate host and in an insect vector. Transitions from one stage to another are accompanied not only by morphological changes, but also by adaptations to the nutritional conditions encountered in the different hosts. Glucose is a major energy source, but amino acid catabolism can also serve to provide energy and other substrates. A unique feature is the compartmentalization of the reactions of the Embden-Meyerhof pathway in organelles referred to as glycosomes [124,125]. The fate of pyruvate then varies between members of the family, between different stages of the same species, and it depends also on the aerobic or anaerobic conditions encountered by the organism [126]. Pyruvate may be completely oxidized to CO<sub>2</sub> by the classical pathway, but one also finds acetate as the end product, and ATP generation from a combination of acetate:succinate CoA transferase activity and the succinate:succinyl-CoA cycle. A detailed discussion of this subject is beyond the scope of this section.

Of special note here is the existence of alternate oxidases in trypanosomatid mitochondria which function in the maintenance of the redox balance in the glyoxisome. Reducing equivalents are moved from the glycosome to the mitochondria via a glycerol-3-phosphate/ dihydroxyacetone phosphate shuttle, and an FAD-linked glycerol-3-phosphate dehydrogenase is used to donate electrons to the UQ/UQH<sub>2</sub> pool. UQH<sub>2</sub>

may then be oxidized via the classical electron transport chain coupled to ATP production, or by a plantlike alternative oxidase. Thus, the long-slender bloodstream stage of *T. brucei* does not possess the usual complex III/complex IV respiratory chain, and uses the cyanide-insensitive oxidase exclusively [126]. In the procyclic (insect stage) of *T. brucei* cytochromes and oxidative phosphorylation are observed readily, although there has been a debate over the apparent absence of a rotenone-sensitive NADH-dehydrogenase. More recent experiments suggest that procyclic *T. brucei* have a proton-translocating complex I, but with a subunit composition different from the mammalian complex and a decreased sensitivity to rotenone [127].

## 5.4 THE CHEMIOSMOTIC HYPOTHESIS

### 5.4.1 The Mitchell Hypothesis

We return now to the question raised earlier in this chapter about how the combustion of foods to  $\text{CO}_2$  and water and the accompanying free energy change can be exploited in biological systems to do beneficial work in muscular contraction, biosynthetic reactions, and conductance of nerve impulses, for example. The experiments and ideas of H. Kalckar and F. Lipman had focused attention on ATP as the most useful and versatile intermediate in this interconversion of energy, and therefore the problem could be restated: how is respiration and the oxidation of carbon compounds coupled to the synthesis of ATP? (A large volume of research in bioenergetics and biochemistry is condensed in these simple summarizing statements.)

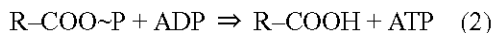
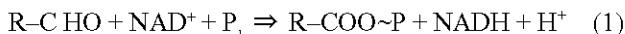
A theoretical foundation for the solution to this problem was proposed in 1961 by P. Mitchell [128], but the idea was so revolutionary at the time that it took another 10 to 15 years to convince most of the influential skeptics (as opposed to those who did not understand it at all) of its validity, and in turn, of its extremely broad applicability to a wide variety of basic biological mechanisms in oxidative phosphorylation, photosynthesis, and active transport. Other ingenious models had been proposed, and they co-existed for a while with the "Mitchell hypothesis." Models suggest experiments, and with time experimental observations lead to models being discarded, modified, or even combined. Broadly speaking, three competing models existed during the early 1960s, and for convenience they can be named after their major proponents:

1. Slater's chemical coupling hypothesis
2. Boyer's conformational coupling hypothesis
3. Mitchell's chemiosmotic hypothesis

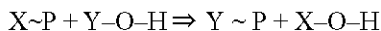
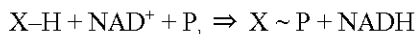
The first was essentially an extrapolation of insights gained from the understanding of substrate level phosphorylation (see below). The second was prompted by the failure to find experimental support for the first, and the third was a radically new synthesis of ideas.

When the glycolytic pathway had been elucidated, several energy conserving reactions had been established in which a free energy release resulting from an oxidation is

coupled to the synthesis of ATP. The oxidation of glyceraldehyde-3-phosphate by  $\text{NAD}^+$  converts an aldehyde to an acid group, but at the same time the enzyme glyceraldehyde-3-phosphate dehydrogenase “activates” inorganic phosphate by linking it to the acyl group. The reaction involves an acyl thioester intermediate which can be attacked by inorganic phosphate acting as a nucleophile. In a subsequent reaction this phosphate was transferred from the high energy mixed anhydride to ADP to form ATP. Thus:



Group transfer reactions and the idea of high energy phosphate bonds ( $\text{X} \sim \text{P}$ ) are illustrated here. The oxidation of  $\text{X}-\text{H}$  and activation of inorganic phosphate lead to the formation of  $\text{X} \sim \text{P}$ . The phosphate can then be transferred to an acceptor to form a lower energy phosphate bond



where  $\text{Y}-\text{O}-\text{H}$  could be a sugar, an alcohol, or ADP.

For almost two decades several prominent biochemistry laboratories sought to identify the mysterious compound  $\text{X} \sim \text{P}$ , which was postulated to be the intermediate trapping the free energy from mitochondrial electron transport (and oxidations). A mind-set derived from classical biochemistry required the presence of a compound,  $\text{X} \sim \text{P}$ , even if very short lived, as part of the mechanism of attaching inorganic phosphate to ADP. It should be pointed out that “ $\text{X}$ ” could also represent a side chain of an amino acid or one or more peptides in the electron transport chain. For a while, phosphorylated histidine was a favorite, but in the end all efforts to identify such a chemical intermediate failed completely.

In the conformational coupling hypothesis the energy from oxidations and electron transport was thought to be “captured” in a high energy conformational state of a protein (ATP synthase) which could then be used to drive the synthesis of ATP. It is noteworthy that a model for conformational coupling included two alternating sites for ADP and  $\text{P}_i$  binding, ATP synthesis and release [129]. Conformational changes are still very much part of the mechanism of electron transport and proton pumping. On the other hand, it is true that “. . . the word conformational, applied to proteins, acquired a kind of magical significance, enabling proteins to accomplish anything (conveniently without the need to specify any biochemical mechanism) . . .” [130]. The fundamental question is concerned with the coupling of electron transport and ATP synthesis, and conformational changes in the complexes of the ETC and in the ATP synthase are not physically coupled.

P. Mitchell was able to approach the problem from a radically different direction. Restating a view expressed by van't Hoff, he has pointed out that “imagination and shrewd guess work are powerful instruments for acquiring scientific knowledge

quickly and inexpensively. . . .” In his view of science, adopted from Popper, preconceived models are subject to constant experimental testing, . . . “thus detecting and discarding the concepts that are false and retaining concepts that show by their survival that they are factually serviceable because they represent reality as far as it is known.” Several concepts merged in an inspired hypothesis increasingly supported by accumulating experimental evidence, which was almost nonexistent at the beginning. Mitchell and his colleagues have published numerous papers and reviews [84,128, 130–137]; a very readable and comprehensive review of the chemiosmotic hypothesis and the evolution of the ideas leading to it is the Ninth Sir Hans Krebs Lecture delivered in 1978 [130]. It is probably fair to say that, although Mitchell contributed to the experimental support of his hypothesis, his brilliant idea provoked many other talented individuals either to support or challenge it, and in the end it was verified as another major landmark in the intellectual landscape of bioenergetics.

The first idea was that an enzyme embedded in a membrane in a unique orientation might catalyze a reaction by accepting substrates on one side of the membrane and release the products on the other side. This concept was referred to as “vectorial metabolism.” It arose from two major biochemical mysteries: oxidative phosphorylation, and the linkage between metabolism and transport. Even a “simple” group-transfer reaction occurring in a single aqueous phase was already considered a “chemiosmotic” process by Mitchell, because “the group-translocation pathway represents the field of action of a real through-space-force corresponding to the chemical potential gradient.” Such microscopic osmotic processes could be converted to a macroscopic osmotic processes if the enzyme was “appropriately plugged through a membrane.” Therefore, the free energy change associated with such a reaction might be used to drive the accumulation of a molecule or ion against a concentration gradient, that is, an enzyme could under some conditions work like a pump. Third, a concentration gradient of a solute across a membrane could be considered as a stored form of chemical free energy. If ions were the solutes in question, a concentration gradient might also be manifested as a membrane potential, and the membrane assumed properties similar to a charged capacitor. These ideas had been theoretically explored by Nernst. As Mitchell recalls [130], his ideas were further influenced by the proposal of electrochemical fuel cells made already in 1839 by W.R. Grove, and by E.A. Guggenheim’s thermodynamic treatment of electrochemical cells and electric circuits, splitting chemical reactions spatially into two half reactions, connected internally by a specific conductor of one chemical species, and connected by an outside circuit conducting another chemical species.

Progress with a number of simpler experimental systems added support to many of these individual concepts. Neurobiologists started to understand membrane potentials, sodium and potassium gradients across a membrane, and the mechanism of generating an action potential. Since the lipid bilayer is completely impermeable to ions, specific integral membrane proteins had to be identified to act as ion pumps and regulated gates whose combined action could explain such phenomena first for excitable membranes and then in more general situations. The sodium potassium pump of the plasma membrane and the Ca pump of the sarcoplasmic reticulum became classic examples of ion pumps which could use the hydrolysis of ATP to pump ions against



their concentration gradients and against an electrical gradient (active transport). These reactions were stoichiometric: a mole of ATP hydrolyzed was coupled to the transfer of a fixed number of ions across the membrane (3Na<sup>+</sup> out of the cell, 2K<sup>+</sup> into the cell in the case of the Na K pump). Later, simple proton pumps were discovered in the membranes of lysosomes, for example. We still do not understand at an atomic level of resolution how such pumps translocate ions across a membrane, but the pumps have been purified, and reconstituted in pure synthetic membranes and vesicles. The corresponding genes have been cloned and sequenced. The corresponding amino acid sequences have led to predictions of structure and correlations of structure with function. It became apparent and demonstrable that such pumps could operate in reverse following the simple law of mass action. An artificially high, experimentally established gradient could drive a reaction in the reverse direction and synthesize ATP from ADP and inorganic phosphate.

Another important concept contributed by Mitchell was the distinction between chemiosmotic and purely osmotic reactions. An ATPase pumping ions is an example of the former. Symport and antiport are examples of the latter, and they describe the coupled translocations of unrelated solutes across a membrane catalyzed by a porter: both solutes moving in one direction (sym), or two solutes moving in opposite directions (anti). The exchange of ADP and ATP across the mitochondrial membrane is a well known, tightly coupled antiport system, while the uptake of glucose into intestinal mucosa mediated by Na<sup>+</sup> ions is one of the early examples of a symport system. These coupled reactions are not only vectorial, but also involve stoichiometric amounts of each participating solute. One can also “couple” a chemiosmotic reaction catalyzed by one enzyme to an osmotic reaction catalyzed by a spatially distant second enzyme: the Na<sup>+</sup> ion gradient established by the Na<sup>+</sup>/K<sup>+</sup> ATPase can be used to transport glucose into a vesicle or cell by the Na<sup>+</sup>/glucose symporter. From such insights it is not too far to realize that a proton gradient established by the electron transport chain could drive ATP synthesis by a biochemically and spatially distinct complex in mitochondria.

As more and more experiments contributed powerful support for the basic ideas about ion and electrical gradients across membranes, and about active transport (pumps), or pumps running in reverse to make ATP, experimental systems applying these notions to mitochondria and chloroplasts were developed and perfected as well. Significantly, P. Mitchell himself stated that the chemiosmotic hypothesis was actually a by-product of the chemiosmotic concepts of group translocation and vectorial metabolism [131]. Two facts should be re-emphasized at the outset: The ATP synthase of mitochondria resembles a simple proton pump (e.g., in lysosomes) only superficially. Its structure is significantly more complex, as will be described below. Also, pumping protons out of the matrix by electron transport through metal ion centers in the ETC is a very complex system, with the only counterpart in chloroplasts and bacterial membranes.

A second instructive and influential model system developed in the 1970s was the purple membrane bacterium *Halobacter halobium*. Phosphorylation was driven by light energy absorbed by a photopigment soon identified as bacteriorhodopsin. Bacteriorhodopsin was shown to be a light-driven proton pump. A proton gradient in the

dark could drive the synthesis of ATP in *Halobacter halobium*. In combination these two observations suggested that light energy was used to establish a proton gradient across the bacterial membrane (high on the outside, low on the inside), and a proton pump running in reverse was used to make ATP. The proteins involved were distinct and physically separable from each other. On the one hand, this system is clearly an illustration of the importance of a conformational change in a protein playing a role in energy conversion. The photon absorbed by the prosthetic group retinal triggers an all-trans  $\Rightarrow$  11-cis isomerization and hence a conformational change in the bacteriorhodopsin in the membrane. The conformational transitions accompanying excitation and return to the ground state are coupled to proton translocation. On the other hand, the conformational change is not linked directly to phosphorylation of ADP, as in the simple conformation-coupling hypothesis.

In the same vein, it is now clear that the passage of an electron through the various redox centers of complexes I, III, and IV may be accompanied by conformational changes throughout the complex (one or more subunits), and at least in the case of complex IV, these conformational transitions are believed to drive proton translocation. These changes need not be very dramatic and may involve only a limited number of side chains along a "channel" for proton translocation. The situation is potentially more complicated and controversial for complexes I and III, because the participation of ubiquinone creates additional theoretical possibilities for proton translocation (see below).

A key aspect of Mitchell's chemiosmotic hypothesis is the requirement that respiratory (and photoredox) chains translocate protons in one direction, while ATP synthesis is driven by proton flow in the opposite direction (Figure 5.19). While self-evident today, a violation of this prediction would have been the end of the hypothesis. Testing the prediction was not trivial, but eventually supportive. A second prediction or requirement has been even more difficult to verify absolutely: the protonmotive systems were predicted to have a characteristic stoichiometry for the number of protons translocated per two electrons passing through the system ( $H^+/2e^- = H^+/O$ ). Similarly, the ATP synthase was postulated to have a characteristic  $H^+/P$  stoichiometry, that is, protons required to make one ATP from ADP and  $P_i$  [130,131]. Two electrons are used here because two are needed per oxygen atom converted to water. Combining these ratios leads to the P/O ratio, a parameter which is experimentally accessible. Attempts to measure it have been made by numerous investigators. The initial measurements seemed to support Mitchell's original predictions, and they have found their way into the textbooks.

With NADH as the substrate, P/O ratios of 3 have been reported, and with succinate the P/O ratio is 2. The  $H^+/P$  stoichiometry for the ATP synthase is typically stated to be 3. Direct measurements of  $H^+/2e^-$  or  $H^+/O$  ratios are more complicated, and subject to potential errors. The method is the "respiratory pulse method" which measures a pH change in a mitochondrial suspension in the presence of a specific substrate following a brief pulse with a known quantity of oxygen. It measures the sum of activities for all redox loops activated by the substrate. When decomposed for the individual loops, the presently accepted  $H^+/2e^-$  ratios are indicated by the equations given for the individual complexes

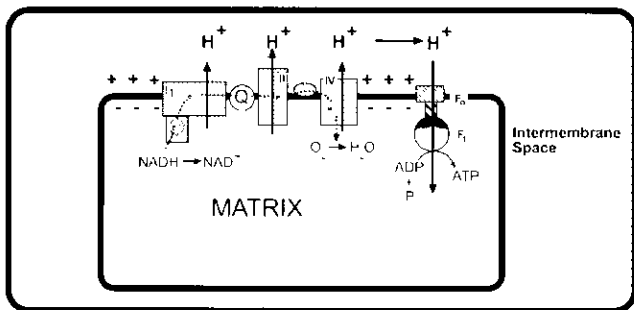
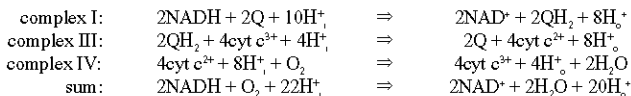


Figure 5.19 Schematic illustration of the chemiosmotic hypothesis.



Several comments are in order. It was recognized by Mitchell and others that mitochondrial membranes are not perfect insulators for protons, as viewed in the ideal model system, but instead have a variety of "proton leaks." These include specific symport or antiport systems, or as yet poorly specified leakage channels. For example, the ADP/ATP antiporter has been reported to be electrogenic [138], while the phosphate/ $\text{OH}^-$  antiporter transports one proton into the matrix [139]. Thus, the export of one ATP is coupled to a net import of one proton, and the  $\text{H}^+/\text{P}$  ratio has to include this reaction.

Over the years there have been numerous reports reporting P/O ratios which were less than the integral values of 3 for NADH oxidation and 2 for succinate oxidation ([140] for a listing of additional references). It is therefore very important to emphasize that such observations in no way contradict the fundamental postulates of Mitchell's hypothesis. Each redox site and the ATP synthase carry out vectorial metabolism, and, for example, the number of protons translocated per electron pair passing through complex IV is stoichiometrically fixed. However, proton pumping by the electron transport chain and ATP synthesis are physically distinct processes, and if the protons find other paths back into the mitochondrial matrix, ATP synthesis will be less than what would be maximally possible in a system with a perfectly insulating membrane, the ETC and the ATP synthase. A recent re-evaluation of energy loss due to proton leakage has been made by Hinkle and colleagues [140]. They also investigate potential systematic errors in typical experiments, for example, ADP contaminated with AMP, oxygen consumption during "state 3" and in the transition to "state

4" (see Section 5.6), calibration of the oxygen electrode. Thus, both systematic experimental errors and theoretical considerations now make it possible to obtain fractional P/O ratios that are rational and do not violate the Mitchell hypothesis.

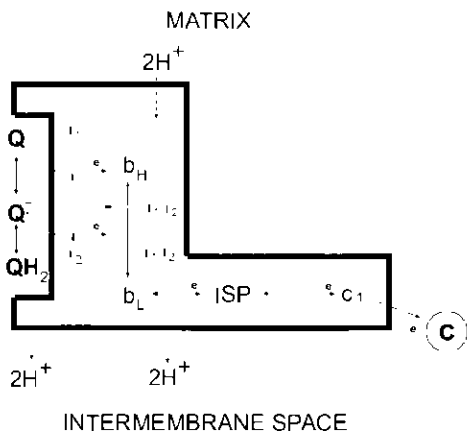
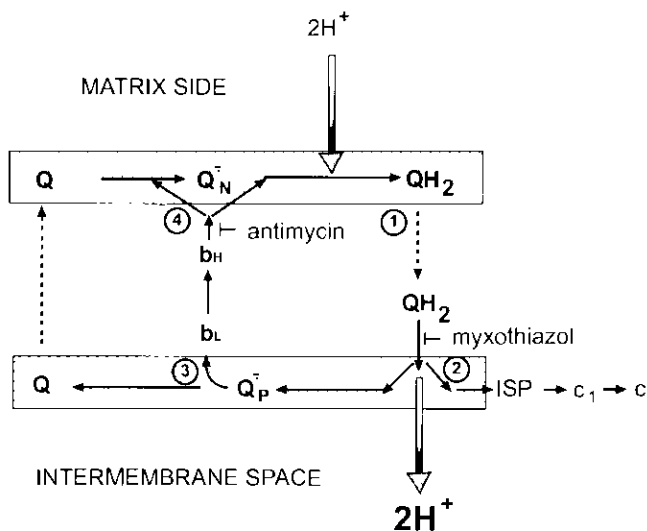
As reviewed by Hinkle and coworkers [140], the  $H^+/P$  ratio has also fluctuated somewhat over the years, ranging from 3 to 4 for synthesis and transport. It can be measured by measuring the free energy change for ATP synthesis and the electrochemical proton gradient (pH corrected for electrochemical component).

Finally, the  $H^+/O$  ratio at each of the three coupling sites has been a contentious issue for some time, but a consensus appears to have been reached, and some semantic confusion has been removed. The latter arises because at each coupling site there is proton translocation as well as proton absorption or formation by the overall reactions, and therefore the count has to be made consistently. This leads to the Q cycle, the last significant mechanistic component of the ETC, also proposed originally by Mitchell [84], and critically reviewed and modified recently by Trumpower [87,141].

### 5.4.2 The Q Cycle

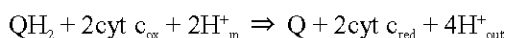
The role of ubiquinone as a mobile carrier between complexes I and II and complex III has been mentioned before, but its role in proton translocation has not been emphasized so far. The structure and various oxidation states of ubiquinone are shown in Figure 5.6. Two properties of ubiquinone are significant for the understanding of the Q cycle. First, the carrier is lipid-soluble in the oxidized and the reduced form. The quinone moiety can therefore diffuse from one side of the membrane to the other and carry hydrogen atoms with it. Second, the unprotonated semiquinone is a relatively stable intermediate, which itself cannot diffuse through the membrane, because of its negative charge. There are 10 isoprenoid units in the naturally occurring ubiquinone, making an aliphatic carbon chain of forty carbons with regularly spaced double bonds and methyl side chains. Such a chain is much longer than what is required to span a lipid bilayer, and the usual representation of a ubiquinone molecule diffusing through a schematic membrane many diameters wider than the Q is likely to give a totally wrong impression of the "diffusion" of Q "across" the membrane. Ignoring this problem for the moment, the complete Q cycle actually is the sum of two cycles, shown in Figure 5.20A.

A consideration of the Q cycle is conveniently started with reduced  $QH_2$  supplied by complex I or complex II on the matrix side of the membrane (step 1). Diffusion across the membrane brings it to the other side and in a position to give up two protons to the intermembrane space, passing one electron to the iron-sulfur protein (ISP, Rieske protein) of complex III (step 2). This electron is passed on to the  $cyt\ c_1$  of the complex III and finally to cytochrome c. The semiquinone in the unprotonated form gives up a second electron to the first of two hemes,  $b_L$ , and becomes oxidized to Q (step 3); the electron is passed to the second heme  $b_H$ . Q diffuses back to the matrix side, and there it is reduced to the semiquinone by the heme  $b_H$  (step 4). During this first half of the Q cycle the net reaction is the oxidation of  $QH_2$  to the semiquinone, the reduction of one cytochrome c, and the transfer of two protons to the intermembrane space. A repeat of the steps 1–3 starting with  $QH_2$  generates Q, another reduced



**Figure 5.20** A: Another view of the proposed Q cycle by Trumpower and colleagues (From Reference [87]) B: A modification proposed by Matsumo-Yagi and Hatefi. (From Reference 142)

cyt c, a reduced heme  $b_H$ , and releases two more protons in the intermembrane space. At this time, however, the heme  $b_H$  is transferring its electron to the semiquinone, accompanied by the pickup of two protons from the matrix side to form ubiquinol. The summation of the two series of reactions is



The net reaction is that four protons are translocated to the outside (intermembrane space) for every pair of electrons passing through complex III from  $QH_2$  to cytochrome c [141] (Figure 5.20A).

The protonmotive Q cycle originally proposed by Mitchell and later modified by Trumpower has made it into the standard textbooks, and appears to have gained general acceptance. Arguments in support of this formulation, especially with reference to the two inhibitors antimycin and myxothiazol binding at the “center N” and the “center P,” respectively, have been critically reviewed by Brandt and Trumpower [141]. Nevertheless, some doubts about its validity have been raised by some recent experiments by Matsuno-Yagi and Hatefi [142,143], again with special attention focused on observations made with the two inhibitors. Although the Q cycle hypothesis can account well for a number of observations, and although these new experiments also leave some unanswered questions, they lead to a provocative alternative model of  $QH_2$  oxidation and proton pumping by complex III. The novel aspects of this model are the following: First, the Rieske protein does not receive electrons from ubiquinol directly, but from one of the hemes of cyt b. Second, the two inhibitors, although they bind to different sites, do not affect the Q cycle at distinct, independent steps, but, by binding allosterically, influence several steps at once. These include electron transfers from the ubiquinol/semiubiquinone to the cyt b as well as electron transfers between the two hemes, and from the hemes to the iron protein. The proposed model (Figure 5.20B) has the attractive feature that the ubiquinol does not have to execute as much motion within the membrane and the complex. It also places the path for proton translocation within the cyt b with its eight transmembrane helices, and removes this function from the mobile carrier ubiquinol.

In summation, it can be stated that Mitchell's chemiosmotic hypothesis has stood the test of time, and therefore it “represents reality as far as it is known.” Its major insight was that a proton gradient across the inner membrane can be set up independently by electron transport (like water moved uphill into a dam by evaporation and condensation), and this gradient can be used to drive ATP synthesis (water running through turbines to produce electricity).

One final point needs to be made more explicitly once again. In an initial presentation of the chemiosmotic hypothesis, the emphasis was on protons, and therefore on the pH gradient across the inner membrane. Since protons are charged, such a gradient also creates a membrane potential,  $\Delta\Psi$ . In considering the thermodynamic parameters for ATP synthesis, a driving force is therefore not only the concentration gradient represented by  $\Delta\text{pH}$ , but there is also an electrochemical contribution from moving a positive charge from high to low potential. Other ions contribute to the overall electrochemical potential difference: protons or hydroxyl ions participate in antiport or symport systems, exchanging protons for metal ions, hydroxyl ions for

phosphate, and so on. Thus, the proton electrochemical gradient is conventionally denoted  $\Delta\mu_{\text{H}^+}$ , and its components are  $\Delta\Psi$  and  $\Delta\text{pH}$ . A particularly useful experimental trick has been to use the ionophore nigericin as a  $\text{K}^+/\text{H}^+$  antiporter. It can therefore decrease the  $\Delta\text{pH}$  across the membrane, but not the  $\Delta\Psi$ . In contrast, the ionophore valinomycin is a potassium carrier, and it can collapse the portion of  $\Delta\Psi$  resulting from excess  $\text{K}^+$  on the outside, but not  $\Delta\text{pH}$ . Such experiments will become relevant in the consideration of the control of oxidative phosphorylation and tightly coupled vs uncoupled mitochondria (see Section 5.6).

### 5.4.3 Probing the Mitochondrial Membrane Potential With Fluorescent Dyes

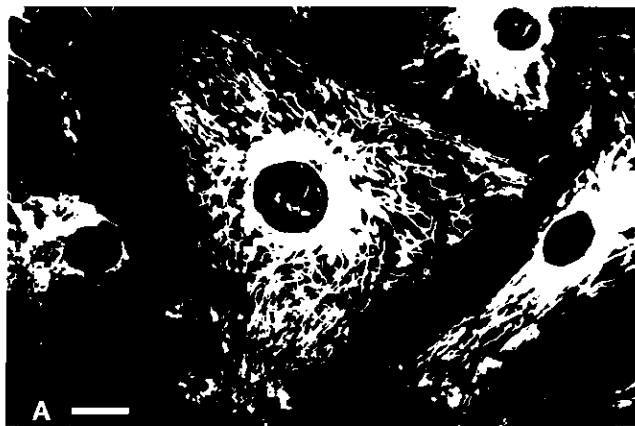
Opposition to Mitchell's ideas arose in part because of misunderstandings about ion transport, membrane potential and the principle of electroneutrality in solutions formulated by classical physical chemistry. The latter principle is never violated in the bulk of the solutions on either side of the membrane. Ion transport separates charges across an extremely thin membrane. These charges separated by a thin insulating layer create a system analogous to a charged capacitor. Because of the extreme thinness of biological membranes an electrical difference of 250 mV across 5–10 nm corresponds to greater than 250,000 V/cm, a very strong electric field by any standard.

The membrane potential is not the same for all mitochondria, or constant at all times for a given mitochondrion. A direct measurement would not only provide strong experimental support for the chemiosmotic hypothesis, but would allow continuous monitoring of the  $\Delta\Psi$  under varying conditions and manipulations of mitochondria. Direct electrophysiological experiments with isolated mitochondria have recently been reported, but they tend to be challenging [144,145]. More convenient methods have been developed over the years. These methods initially gave only qualitative results in most hands, but increasingly quantitative data can be obtained today with appropriate instrumentation. The basis for these optical methods is the existence of dyes which accumulate in mitochondria in a membrane potential-dependent manner. Another advantage of the use of such dyes is that they can be used in living cells to explore mitochondria *in vivo*. The use of dyes to investigate mitochondria goes back to the last century, when mitochondria were first seen without being recognized for their function (see Chapter 1).

A fundamental property of the useful dyes is that they are lipophilic and carry a positive charge which can be delocalized throughout the molecule by resonance structures. They therefore can not only pass through membranes, but they will be actively transported across a membrane with a  $\Delta\Psi$  [146,147]. In the case of mitochondria this leads to their accumulation in the mitochondria. Thus, if the plasma membrane potential can be eliminated, uptake and fluorescence of such a dye in a cell can become a measure of the mitochondrial membrane potential. At the simplest level of analysis one can stain mitochondria and determine their intracellular distribution, their behavior in response to disruptions of the cytoskeleton, or their behavior during cell division [148]. Two types of quantitative measurements should be distinguished: 1) one

can measure total, integrated fluorescence from a fixed number of cells and obtain information about the overall mitochondrial activity in the population of cells; 2) one can monitor individual cells, and even individual mitochondria within a cell to address questions about intercellular and intracellular heterogeneity. The choice of the appropriate dye is an important consideration, and even then very precise quantitative measurements and their interpretation are subject to theoretical limitations which should not be ignored. Experimentally such measurements are not as simple as measuring concentrations from absorbances and the use of Beer's law.

The first dye to be used extensively was rhodamine 123 (Rh123) [149] (Figure 5.21). A serendipitous observation was soon exploited by Chen's laboratory to demonstrate the specificity for mitochondria in living cells. Its uptake into mitochondria was dependent on  $\Delta\Psi$ , since various ionophores and uncouplers known to dissipate the membrane potential could be shown to prevent Rh123 uptake. Similarly, inhibitors of the electron transport chain (rotenone, antimycin, cyanide) can also reduce or eliminate the uptake of Rh123. It was observed that when glycolysis produces sufficient ATP, a membrane potential could still be generated from the reverse reaction of the  $F_0F_1$  ATPase, a reaction which can be inhibited by oligomycin. In the presence of the  $H^+/K^+$  antiporter nigericin, Rh123 could be used to show hyperpolarization of the membrane, since the proton gradient can be converted into a  $K^+$  gradient with less inhibitory effect on electron transport. Thus, Rh123 fluorescence was a faithful indicator of membrane potential in mitochondria. As described in more detail in the original literature (summarized in Reference [150]), the effects of a plasma mem-

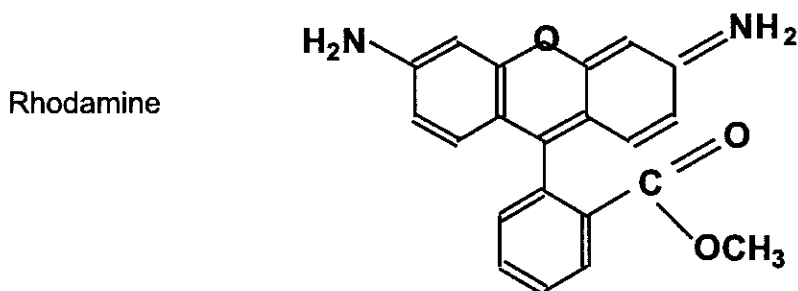


**Figure 5.21** Mitochondria stained with rhodamine 123. (Photograph provided by Dr. Lan Bo Chen.)

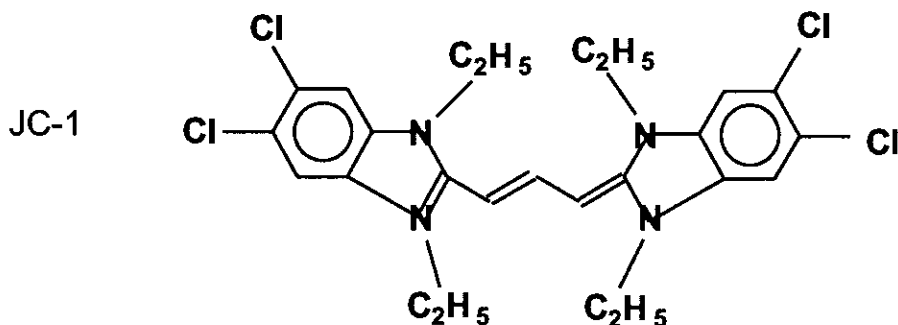


brane potential can be eliminated in  $137 \text{ mM K}^+$ , and for some experiments the inhibitor ouabain has to be included to shut off the sodium-potassium pump in the plasma membrane

Literally thousands of different dyes from the color photography industry were tested. Several others are discussed below. An excellent source of up to date information on "MitoTracker" probes is the Handbook of Fluorescent Probes and Research Chemicals (Molecular Probes, Eugene, OR). However, Rh123 (Figure 5.22A) has remained a favorite for two main reasons. It appears to be the least toxic to cells, measured by a variety of criteria: survival after weeks of exposure, DNA, RNA, and protein synthesis, operation of the secretory pathway, maintenance of the cytoskeleton and cell motility. It may be of special interest that it appears more toxic to cancer cells. It also appears to be unique in being unable to stain mitochondria in the absence of  $\Delta\Psi$ , that is, when azide and oligomycin are used to shut down all mechanisms to produce it, while most of the other dyes tested still give some staining of mitochondria. Differences in lipophilicity and the presence of special lipids within the inner



5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide



**Figure 5.22** Structures of rhodamine 123 and the dye JC-1

membrane (cardiolipin) have been offered as explanations [150], but the result remains partially empirical at this time. A word of caution is in order. Studies have clearly shown that these dyes may interact with individual complexes of the electron-transport chain, for example complex V (W. Allison, personal communication). Thus, inhibitory effects of these dyes in long term studies should not be ignored.

The concentrations of dyes, the length of exposure of the cells to the dye, the excitation wavelength and the emission spectrum are all parameters which have to be considered. For Rh123 the typical conditions are: 0.1 mg/ml for 3 hours, excitation by blue light (485 nm), and a greenish fluorescence is measured with the filter normally used for fluorescein dyes [150]. For details the original papers should be consulted.

Experiments with Rh123 have shown that all mitochondria in a given cell are homogeneous with respect to fluorescence intensities, i.e. have identical membrane potentials, regardless of shape, size, intracellular location. On the other hand, in a population of cells, significant differences in  $\Delta\Psi$  between individual cells have been observed [150] (see below). Not surprisingly, mitochondria in cardiac and skeletal muscle cells have the highest membrane potentials, followed by smooth muscle, macrophages, hepatocytes, fibroblasts, resting neuronal cells, glial cells, and keratinocytes. The lowest  $\Delta\Psi$ s were found in resting T and B lymphocytes. Differences have been observed between resting cells and rapidly proliferating cells, and also in the course of cell differentiation. The most dramatic change occurs when myoblasts differentiate into myotubes, suggesting an activation of mitochondria which is still under active investigation. Another significant change in Rh123 fluorescence and hence  $\Delta\Psi$  is observed at some stage in the progression of a cell toward apoptosis (see Section 7.4), when the mitochondrial permeability transition creates large pores for the dissipation of all ion gradients. A large number of tumor cells have been investigated and compared with their normal counterparts [150]. In general, carcinoma cells were found to have higher membrane potentials, but the significance is still not completely clear. It may be related to their proliferative potential.

In recent years several other dyes have found applications for greater sensitivity and discriminations between mitochondria within a given cell and even between regions of the inner membrane in a single mitochondrion. The dyes can be divided into two classes, fast dyes and slow dyes. An example of the first is Rh123, a molecule which can partition into and across the lipid bilayer in the presence of an electric field and respond almost instantly to a change in membrane potential by a change in spectroscopic characteristics (quantum yield, absorption or emission spectrum). The slow dyes form aggregates in specific environments, again accompanied by dramatic changes in optical properties compared to the monomer in solution. One of these is the cyanine dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolecarbocyanine iodide, mercifully referred to as JC-1 [151] (Figure 5.22B). It was initially used as a sensitizer for silver-halide-based photography. Theoretical treatments attempting to explain the aggregation and spectral changes are available [see Reference 151 for references]. Several aspects of the uptake and behavior of JC-1 by isolated mitochondria and intact living cells were examined [151]. When coupled, energized mitochondria were titrated with JC-1, an absorption peak corresponding to the monomer (527 nm—green) was observed first which was not affected by ma-

nipulations of the membrane potential. It can be taken as a measure of mitochondrial volume [152]. At higher concentrations, a second peak (590 nm—red) appeared which corresponds to J-aggregate formation, and this J-aggregate fluorescence was proportional to the membrane potential over the range of 30–180 mV. Less quantitative estimates with a fluorescence microscope are based on mitochondria appearing orange in the energized state and green in the uncoupled state. Most surprisingly, within a certain range of average  $\Delta\Psi$ , mitochondria were detected with apparently heterogeneous staining, indicative of local fluctuations in  $\Delta\Psi$  within a single mitochondrion. Similarly, J-aggregate formation and red fluorescence in mitochondria of living cells were sensitive to the electrochemical gradient, that is, the aggregates failed to appear in the presence of either CCCP, dinitrophenol, or azide plus oligomycin, or they could be dissociated when these uncouplers or inhibitors were added later. Combinations of ionophores were used to show that aggregate formation was sensitive to  $\Delta\Psi$  but not to  $\Delta pH$  alone.

Significantly, a number of different cell types examined exhibited both intercellular and intracellular heterogeneity in staining with JC-1 which had not been detected previously with Rh123 or other cyanine dyes [e.g., 153]. The explanation offered was that detection systems in the past (photography, the human eye) may have been used outside of the linear response range, that is, were saturated and hence incapable of discriminating local differences. Therefore, even intracellular heterogeneity must now be taken seriously, and an explanation must be sought in terms of different microenvironments for mitochondria within a cell, of the type already described by sensitive determinations of  $Ca^{2+}$  gradients. Dynamic, multichannel continuous recordings may in the future be able to address these questions.

An even more challenging problem arises from the observation of low and high membrane potentials (or at least an uneven distribution of J-aggregates) within a single mitochondrion [151]. Proton diffusion is extremely rapid, and localized proton circuits or “respiration hot spots” within a mitochondrion are almost certainly transient phenomena. How can a slow dye respond to these, and how are such states maintained at least during the interval required for photography or other detection methods?

## 5.5 ATP SYNTHASE ( $F_1F_0$ -ATPASE)

### 5.5.1 Introduction

Reference has been made repeatedly to a distinct complex (V) playing a crucial role in oxidative phosphorylation. An enzyme had to be responsible for the synthesis of ATP from ADP and inorganic phosphate. Somehow, this endergonic reaction had to be coupled to redox reactions and electron transport. The acceptance of Mitchell's chemiosmotic hypothesis and the elucidation of the biochemical and structural properties of this enzyme complex evolved somewhat in parallel, and today we understand that a physically separable complex can catalyze this reaction driven by a proton gradient, which can be set up artificially across a vesicle membrane simply by the choice

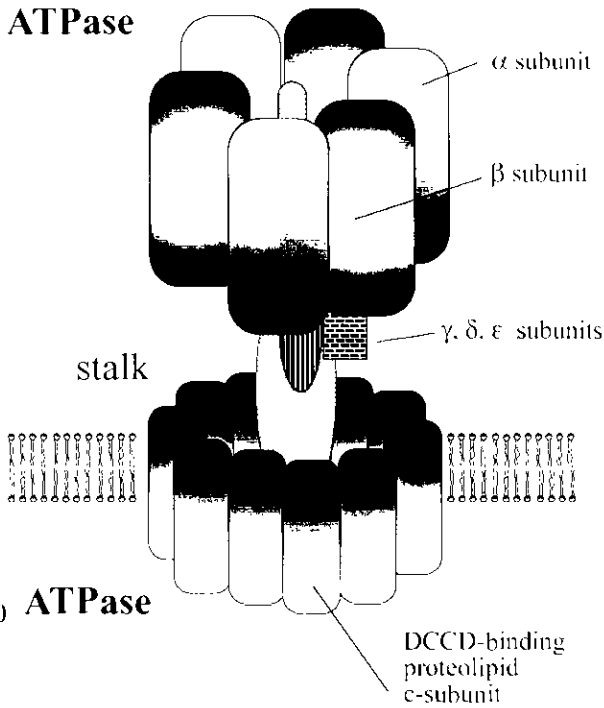
of buffers, or by the incorporation of a light-driven proton pump from the purple membrane bacterium *Halobacter halobium*. ATP synthase was first “seen” by electron microscopists around 1962. Lollipoplike structures were identified by their spherical heads on the matrix side of the inner membrane, and these were attached to the membrane by a slender stalk. Pioneering work in E. Racker’s laboratory had succeeded in dissociating the heads from the stalk. The heads were soluble in water, and could be reassociated with the membrane in the presence of an essential stalk protein known as the “oligomycin sensitivity conferring protein” (OSCP). OSCP had first been isolated and characterized in the laboratory of Tzagoloff [154–156]. Inhibition of the ATP synthesis by oligomycin had become a diagnostic test for this mitochondrial enzyme, and therefore the physiological significance of the required reassembly factor was apparent.

The complete complex was separated in the fractionation scheme developed by Hatefi [9,10], and the combined efforts of these early investigators led to the now well-established separation of the complete complex V into a soluble  $F_1$ -ATPase, and an insoluble membrane complex referred to as  $F_0$ . The  $F_0$  complex has the ability to translocate protons across the membrane from their high potential on the outside, and when coupled to the  $F_1$  subcomplex, ATP synthesis can be achieved.

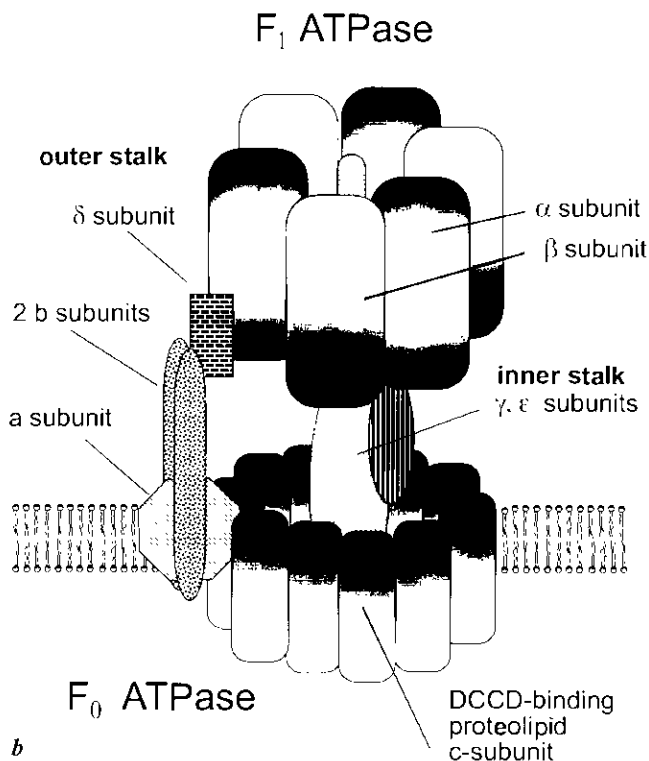
It was quickly apparent that this structure is significantly more complex than the first ion pumps being characterized, the  $\text{Na}^+/\text{K}^+$  pump in the plasma membrane consisting of two subunits ( $\alpha\beta$ ), and the  $\text{Ca}^{2+}$  pump in the sarcoplasmic reticulum. The  $F_1$ -ATPase has five different peptides,  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ , present in the ratio 3:3:1:1:1 (Figure 5.23A). The mitochondrial  $F_0$  subcomplex (Figure 5.23A) has a variable number of different subunits depending on the species (11 in mammals including the inhibitory factor  $\text{IF}_1$ ), of which three essential proteins are homologous and functionally related to the three proteins in the bacterial complex, and the remainder are accessory proteins necessary for assembly, stabilization and control. All three core proteins are encoded by the mitochondrial genome in *Saccharomyces cerevisiae* and plants, while one of these genes has been transferred to the nucleus in animals and fungi. The establishment of the stoichiometric ratios for the  $F_0$  subcomplex peptides proved to be quite challenging [157]. Three distinct subunit types (a, b, c) are present in the ratio  $\text{ab}_2\text{c}_n$ , where different authors have reported different values for n, but not greater than 12.

In the literature on this complex, and especially in reviews, reference is frequently made to information from mammalian mitochondria, yeast mitochondria, chloroplasts and the *E. coli* complex in the inner bacterial membrane. The nomenclature is not yet completely uniform, and there is potential for some confusion. For example, the  $F_1$ -ATPase in all examples has five subunits ( $\alpha\beta\gamma\delta\epsilon$ ), but the mitochondrial  $\epsilon$ -subunit has no counterpart (sequence, function) in either chloroplast or *E. coli*  $F_1$  or  $F_0$ , and the  $\delta$  subunits of the bacterial and chloroplast  $F_1$ -ATPase are related to the mitochondrial OSCP which is generally counted among the  $F_0$  stalk peptides. The mitochondrial  $\delta$  and the bacterial  $\epsilon$  subunits are related. The a-, b-, c- subunits of the  $F_0$  hydrophobic sector in mitochondria and bacteria are called subunits 6, 8, and 9 in yeast. Table 5.2 is an attempt to make the cross-referencing easier in the discussion to follow.

## $F_1$ ATPase



More than three decades after its original isolation by Racker's group. [For an account of the early work, see Reference 158]. The studies on the bovine  $F_1$ ATPase culminated in 1994 with the publication of the structure at 2.8 Å resolution by Walker's group at Cambridge [159]. This achievement was recognized by a Nobel Prize in 1997. Over the years the structure evolved from being the globular head of a lollipop roughly 100 Å in diameter, to an increasingly refined description of a number of distinct features based on interpretation of data from enzyme kinetics, biochemistry, immunochemistry, cryoelectron microscopy, and others. For example, the subunit stoichiometry and the determination of nucleotide binding sites suggested the presence of a threefold axis of rotation, and it was reasonable to make this axis parallel to the stalk. Now that the crystal structure of this 371 kDa complex is available, the description could go into considerable detail. Reference to schematic diagrams will be invaluable.



**Figure 5.23** A: Schematic representation of complex V. The  $F_1$ -ATPase extends into the mitochondrial matrix and is connected to the  $F_0$  complex in the inner membrane by a stalk. The major peptides associated with each of the subcomplexes are discussed in the text. B: A modified schematic representation of complex V. See discussion in the text and References [161 and 163] for details. With reference to Fig. 23A, the a- and b-subunits have been added to the  $F_0$  subcomplex, and located outside of the aggregate of c-subunits. The interaction of the  $\delta$  (bacterial) subunit with the  $b_2$  complex and the outside domain of an  $\alpha$  subunit is also emphasized.

### 5.5.2 X-ray Structure

The single subunits  $\gamma$  and  $\epsilon$  form a structure that interacts with components of the  $F_0$  complex on one side and with the  $\alpha$  and  $\beta$  subunits of the  $F_1$  on the other, thus being part of the stalk seen by electron microscopy. The N- and C-terminal segments of the

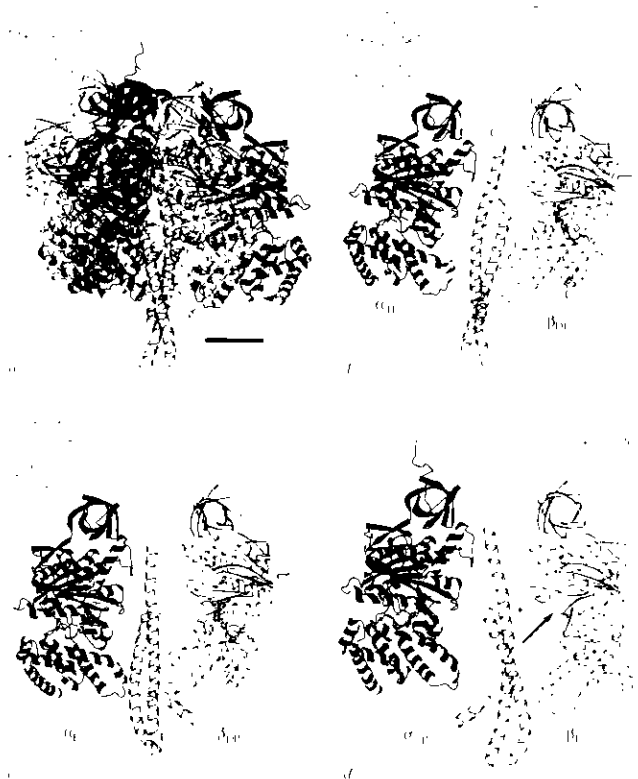
TABLE 5.2 Comparison and nomenclature of complex V subunits in various organisms

| Bacteria ( <i>E. coli</i> )<br>(158) | Animal mitochondria<br>(158) | Yeast mitochondria<br>(159) | Plant Chloroplasts<br>(158) |
|--------------------------------------|------------------------------|-----------------------------|-----------------------------|
| $\alpha$ (55.2)*                     | $\alpha$ (55.1)              | $\alpha$ (55.3)             | $\alpha$ (56.8)             |
| $\beta$ (50.1)                       | $\beta$ (51.6)               | $\beta$ (52.5)              | $\beta$ (53.9)              |
| $\gamma$ (32.4)                      | $\gamma$ (30.2)              | $\gamma$ (30.6)             | $\gamma$ (38.1)             |
| $\delta$ (19.3)                      | OSCP                         | OSCP(20.9)                  | $\delta$                    |
| $\epsilon$ (14.9)                    | $\delta$ (15.1)              | $\delta$ (14.5)             | $\epsilon$ (14.9)           |
| —                                    | $\epsilon$ (5.7)             | $\epsilon$ (6.6)            | —                           |
| —                                    | inhibitor protein            |                             | —                           |
| a                                    | a (ATPase-6)                 | subunit 8 (5.87)            | a                           |
| b                                    | b                            | subunit 6 (27.9)            | b, b'                       |
| c                                    | c (ATPase-9)                 | subunit 9 (7.79)            | c                           |
| —                                    | d                            | d(19.66)                    | —                           |
| —                                    | e                            |                             | —                           |
| —                                    | f                            | b (P25)                     | —                           |
| —                                    | g                            |                             | —                           |
| —                                    | A6L                          |                             | —                           |
| —                                    | F6                           |                             | —                           |

\*The numbers in brackets indicate the molecular mass of the peptides in kDal.

$\gamma$  subunit form extended  $\alpha$  helices forming a left-handed, antiparallel, very loose coiled coil (with an extending C-terminal), and this rod-shaped core has the  $\alpha$  and  $\beta$  subunits arranged alternately around it like the segments of an orange. The  $\delta$  and  $\epsilon$  subunits appear not well-ordered and may be substoichiometric in the crystals [159]. In other words, the purification of the  $F_1$ -ATPase requires a rupture of the stem structure, with the  $\delta$  and  $\epsilon$  subunits becoming randomly distributed and disordered. Thus, their precise orientation and interactions remain to be clarified. It is significant that the coiled coil core with the extended C-terminal helix is somewhat bent and by itself has no threefold symmetry. The symmetrical  $\alpha_3\beta_3$  hexamer therefore is slipped onto an asymmetrical axle, and when the axle rotates (see below), the individual subunits are at any given instant not at equivalent positions. With foresight and good intuition the authors even spoke of a “molecular bearing, lubricated by a hydrophobic interface” [159] when describing the domains surrounding the  $\gamma$  subunit. From a structural or energetic point of view there are no significant barriers against rotation (Figure 5.24).

The positioning of the  $\epsilon$  subunit as part of the stalk is supported by cross-linking studies, and most of the older models had placed the  $\delta$  subunit in a similar position. More recent evidence from studies of the *E. coli* complex in Capaldi’s laboratory [160,161] strongly suggest that this subunit is making contact with the  $\alpha$  subunit of the  $F_1$  complex at a position near the top, distal to the membrane, and at the same time it is connected to the highly elongated b subunits of the  $F_0$  complex extending upward from the base (Figure 5.23B). The relevance of this revised structural arrangement will become apparent when the mechanism of ATP synthesis is considered below.



**Figure 5.24** X-ray structure of the  $F_1$  subcomplex of complex V. A: Ribbon model showing the  $\alpha_3\beta_3$  hexamer and the  $\gamma$  subunit constituting the rotating shaft. B–D: Three different views of the shaft ( $\gamma$  subunit) and side views of two opposite  $\alpha$  and  $\beta$  subunits. The image emphasizes the asymmetry and the different contacts made by each of the six subunits with the central shaft. From Abrahams and colleagues [159], with permission. (See color plates.)

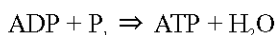
The  $\alpha$  and  $\beta$  subunits are similarly folded: a six-stranded  $\beta$  barrel at the N-terminal is connected through a nucleotide binding domain to a bundle of 7 ( $\alpha$ ) or 6 ( $\beta$ ) helices at the C-terminal. At low resolution one may therefore even think of a sixfold axis of rotation. The nucleotide binding domains are made up of a nine-stranded  $\beta$



sheet with nine associated  $\alpha$  helices, and are recognizable from similarities with other known nucleotide binding proteins such as RecA, and from the presence of typical loops and motifs [159]. They are at the interfaces between the  $\alpha$  and  $\beta$  subunits (i.e., between the slices of the orange). There are three catalytic sites at which nucleotide turnover occurs, and three ATP binding sites where nucleotide binding appears to be required for the maintenance of symmetry and structure, and to prevent abortive complex formation. A proposed physiological regulatory role for these non-catalytic sites is currently without experimental support [162]. Some of the amino acid side chains involved in nucleotide binding had been identified from previous studies such as affinity labeling with 2-azido-ATP, or site-directed mutagenesis (see [163] for earlier references to the primary literature), and the complete structure not only confirmed previous deductions, but allows a detailed reconstruction of all the contacts between the phosphates, ribose and the adenine base [e.g., 162].

### 5.5.3 ATP Synthesis and Catalytic Mechanisms

Before discussing more detailed aspects of the structure, it is necessary to become familiar with the current thinking about the mechanism by which the ATP synthase carries out the simple reaction



on the matrix side of the inner mitochondrial membrane. The problem is that this reaction is endergonic and thus requires an input of energy. The chemiosmotic hypothesis identified a source of this energy and introduced the general notion that this enzyme was a proton pump running in reverse. When this notion was verified experimentally, the challenge became to understand precisely how the coupling of proton flow through the complex and ATP synthesis were achieved. The fractionation of the  $F_1$  ATPase subcomplex was a direct indication that this complex contained the active site for ATP binding, and in the absence of any other driving force it could catalyze the hydrolysis of ATP. The  $F_o$  portion of complex V being an integral membrane complex, it became the obvious choice for a pathway of protons through the inner membrane. The connecting stalk in turn must be the structure which couples these two subcomplexes mechanically and conformationally. The question is how? Several detailed, up-to-date reviews with references to over 300 mostly recent publications have appeared [162,164]. Briefer, elegant reviews and syntheses of many studies can also be found [165,166]. The exploitation of information from the crystal structure promises to be an even more fertile field in the near future.

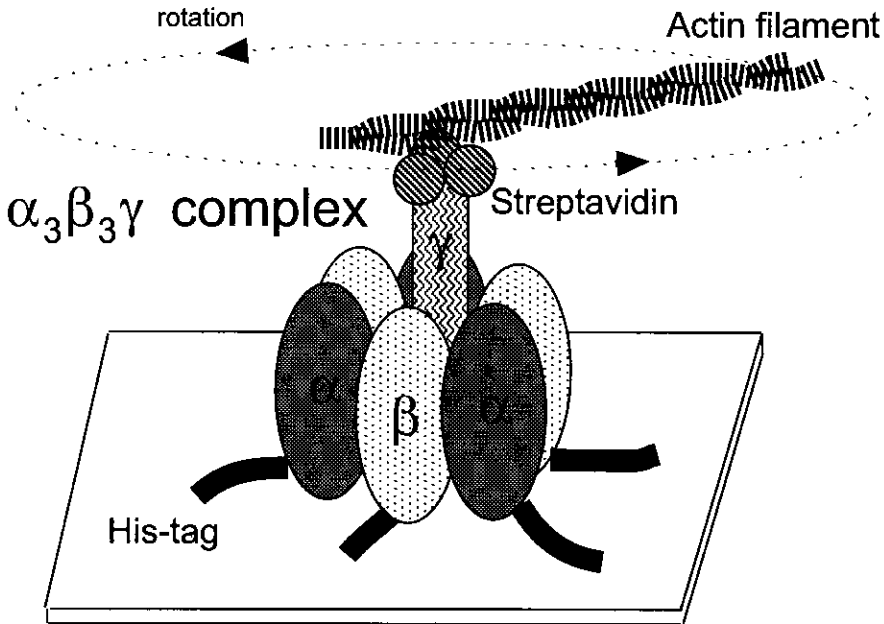
There appears to be a consensus that the binding of ADP and  $\text{P}_i$  at one of the three active sites of the  $F_1$  ATPase provides the energy for combining the two substrates to make ATP. In other words, ATP formation is spontaneous after binding and does not require the input of additional energy. The reaction is a simple reversal of hydrolysis and has no additional covalent intermediates. A nearby glutamate participates in acid catalysis. The electrostatic repulsion between the phosphate groups is

overcome by interactions with amino acid side chains in the active site, and the perfect alignment presumably makes the formation of a covalent bond and the elimination of water entropically favorable. The strong binding energy, however, now makes the release of the ATP unfavorable and slow. It is the release of ATP which is believed to be the step driven by the proton translocation. The release must be triggered by a change in affinity for ATP, which can be brought about by a change in the conformation of the protein subunits. It follows that proton translocation must drive conformational changes. Since all enzyme-catalyzed reactions operate in a cycle with regard to the enzyme, these conformational changes induced by proton flow must also be cyclic.

At this point the subunit stoichiometry of the  $F_1$  complex and the threefold symmetry of the arrangement of the  $\alpha$  and  $\beta$  subunits come into consideration. A "rotating" three-site model was first proposed by Boyer in 1982 [167], as an extension of an "alternating" site model proposed earlier [168,169]. Nucleotide binding studies, chemical probes, ligand/inhibitor binding studies had suggested that the  $\alpha$  and  $\beta$  subunits were conformationally asymmetric, and this concept was fully substantiated by the confirmation of the stoichiometry  $\alpha_3\beta_3\gamma\delta\epsilon$  in the  $F_1$  complex plus stalk. Ignoring the  $\delta$  and  $\epsilon$  peptides for the moment, the coiled coil of the ends of the  $\gamma$  peptide forms an asymmetrical axis on which the  $\alpha\beta$  hexamer is impaled (see above), that is, each  $\alpha$  and or  $\beta$  subunit is exposed to a different surface of this shaft. In the original formulation of the model speculation about rotations of the inner portion of the enzyme relative to the outer portions had already been advanced [164], and it has recently been spectacularly confirmed that a "central rotor of radius  $\sim 1$  nm, formed by the  $\gamma$  subunit, turns in a stator barrel of radius  $\sim 5$  nm formed by the three  $\alpha$  and three  $\beta$  subunits." Rotary motion of the  $F_1$ -ATPase was observed directly (Figure 5.25). In the presence of ATP a fluorescent actin filament attached to the  $\gamma$  subunit was shown to rotate for more than 100 revolutions in an anticlockwise direction when viewed from the membrane side [170].

This elegant and definitive experiment was performed with an  $\alpha_3\beta_3\gamma$  complex from a thermophilic bacterium expressed in *E. coli*. The complex was attached to a microscope slide like an upside-down mushroom, and the stalk ( $\gamma$  subunit) was coupled to fluorescently labeled actin via a multivalent streptavidin molecule binding biotin on the actin and on the  $\gamma$  subunit. The system reconstituted the smallest molecular rotary motor described to date. It is significantly smaller than the only other such rotor described: in the bacterial flagellum a proton flux through the base and socket of the flagellum causes rotations of the flagellum. The flagellar motor has an estimated 100 peptide molecules, but intriguingly, a homologue of the  $\beta$ -subunit of ATP synthase is found in the flagella base complex, and in the future more significant similarities may emerge. In the present case ATP hydrolysis by the  $\alpha/\beta$  subunits caused the  $\gamma$  subunit to rotate, and one can therefore imagine this rotation to be the reverse of the rotation caused by a proton flux driving ATP synthesis. It is noteworthy that the system did not require the  $\delta$  and  $\epsilon$  subunits of the  $F_1$ -ATPase.

The model system, and specifically the direction of rotation, were fully consistent with the "rotating" three site model. Fixing attention on a particular orientation of the central shaft, the rotation will allow three  $\beta$  subunits to interact sequentially with one



**Figure 5.25** Demonstration of the rotation of the shaft ( $\gamma$  subunit) relative to the  $F_1$  complex. Adapted from Noji and colleagues [170].

face of the central shaft. From another point of view, the three orientations of a given  $\beta$  subunit can be shown to correspond to the progression from ATP-occupied  $\Rightarrow$  ADP-occupied  $\Rightarrow$  empty, the reaction sequence expected for the hydrolysis reaction. A rate of hydrolysis of 52 ATPs hydrolyzed per second measured in solution would correspond to 17 revolutions per second, but only about 4 rps were observed. A plausible explanation is the viscous drag on the actin filament, a hydrodynamic load whose effect on the coupling between ATP hydrolysis and rotation remains to be fully investigated. (Interesting comparisons with force/load relationships of other molecular motors such as dynein, kinesin, and myosin can be expected in the future.) The estimated maximum rate of hydrolysis under substrate saturation conditions is 50–100 per second for the bacterial enzyme, but hydrolysis rates by the mitochondrial enzyme may be an order of magnitude higher.

The model system has demonstrated rotation of the  $\gamma$ -subunit relative to the alternating  $\alpha/\beta$  hexamer, which is fixed to the microscope slide. In vivo one can imagine several possibilities. Either the  $\alpha\beta$  hexamer rotates around the fixed shaft formed by the  $\gamma$ -subunit, the  $\epsilon$ -subunit, and the domain of the  $F_0$  complex, which protrudes into the matrix from the inner membrane. Alternatively, both the  $\alpha\beta$  hexamer and the membrane-embedded  $F_0$  complex remain static, and the shaft ( $\gamma$  subunit) rotates. Another model, suggested by Duncan and colleagues [171], has the shaft ( $\gamma$ ) and a complex of c subunits rotating, with the “a” subunit of  $F_0$  placed at the circumference, and the “b” subunit interacting with both the a subunit and  $F_1$ . In this model a, b, and  $F_1$  are

static. In the most recent formulation of the structure of the complete complex V two vertical stalklike structures are postulated. The  $\gamma$  subunit forms the inside "rotor" as described above, but the second stalk formed by a combination of two b subunits and the  $\delta$  subunit acts as the "stator." The end domains of the two elongated b subunits are firmly embedded in the membrane by transmembrane segments (Figure 5.23B). An attachment to the  $\alpha/\beta$  hexamer via the  $\delta$  subunit therefore prevents the  $\alpha/\beta$  hexamer from rotating relative to the base (see [160,166] for the most recent experiments supporting this model of the bacterial complex).

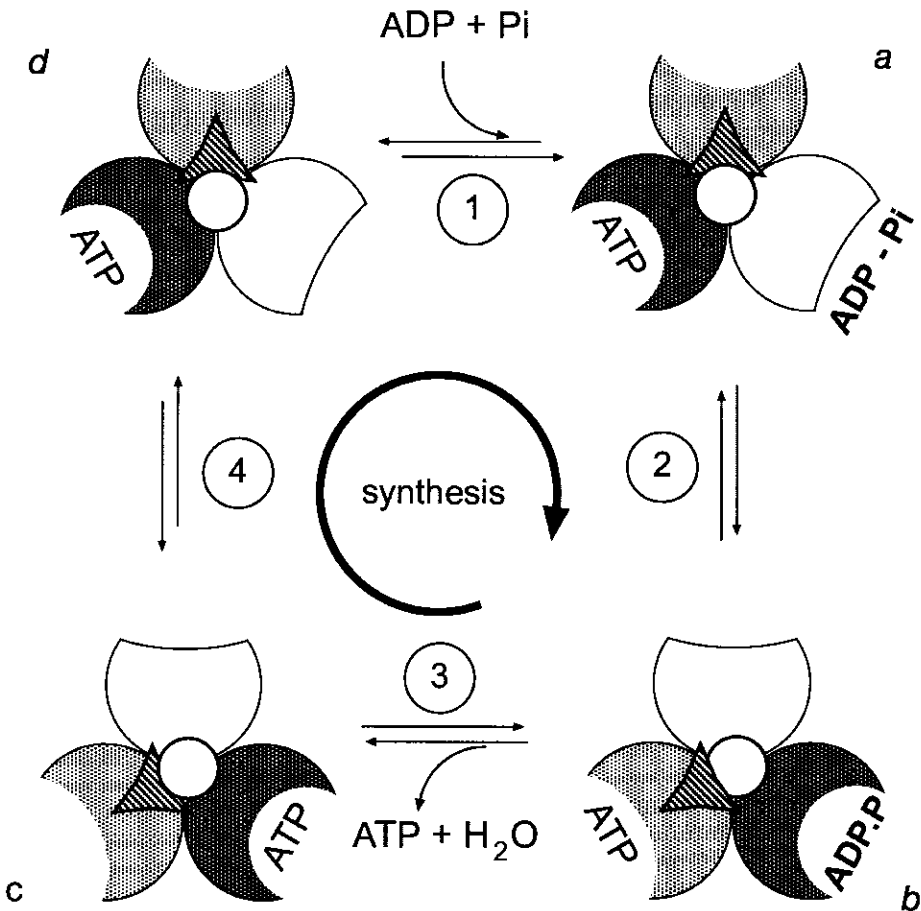
In his authoritative and exhaustive review in 1993 Boyer [164] has provided a tabulation of experimental observations related to the ATP synthase mechanism and provided critical comments on each. An update by the same author incorporating the latest information from the crystal structure has just appeared [172], and highlights in the development of this penetrating and detailed analysis can be found in the acceptance speeches and publications of the 1997 Nobel prize winners in Chemistry. They are testimony to the ingenuity and insights of a large number of investigators keeping pace with or even staying ahead of the structural studies, until this amazing molecular engine and its inner workings are now revealed in exquisite detail. Detailed binding and kinetic studies with nucleotides, nucleotide analogues, and affinity labeling studies had established the presence of three catalytic sites on the  $\beta$  subunit, and an additional three ATP or ADP binding sites at the other interface of the  $\alpha$  and  $\beta$  subunits. Even the catalytic sites are now shown to be formed from residues on both  $\beta$  and  $\alpha$  subunits. The noncatalytic sites have a structural role and are likely to play a part in regulating or modulating the stability and/or the structure of this enzyme [163,164]. Nucleotide exchange at the noncatalytic sites is slow. One is reminded of the role of one nonhydrolyzable GTP in the  $\alpha\beta$  tubulin dimer. Nevertheless, while a physiologically relevant regulatory role has not yet been demonstrated, abolition of the noncatalytic nucleotide binding sites by site-directed mutagenesis has shown that they are not dispensable. Such a modified enzyme ( $\alpha_3\beta_3\gamma$  complex) exhibits an initial ATPase activity, but this activity decays rapidly to zero. This observation has been interpreted to indicate an entrapment of MgADP in a catalytic site [see Reference 173 for additional references to investigations of the specific role of the noncatalytic sites].

Highly informative, crucial results were obtained by measurements of exchange reactions and the distribution of  $^{18}\text{O}$  from inorganic phosphate or ATP under a variety of conditions, or from  $\text{Pi} \rightleftharpoons \text{ATP}$  and  $\text{ADP} \rightleftharpoons \text{ATP}$  exchanges [see references in Reference 164]. These studies were performed with the bacterial complex, with the mitochondrial complex, or with the complex from chloroplasts. The similarities relating to the fundamental principles and mechanisms are overwhelming, and the differences are in some kinetic details.

Once more it should be emphasized that biochemical studies were constantly reviewed and tested for consistency with the structural studies. The clarification of the stoichiometry (for the  $F_1$ -ATPase) was followed by crosslinking studies under various conditions, demonstrating conformational changes associated with the presence of, for example,  $\text{Mg}^{2+}$ -ADP in the catalytic site, contrasted to the presence of the nonhydrolyzable ATP analog AMP-PNP. When the  $\beta$  and  $\gamma$  subunits were crosslinked, en-

zyme activity was severely inhibited, a result now fully explainable in terms of the rotary motion associated with one cycle of activity.

A schematic representation of the three site model is shown in Figure 5.26 below. In the absence of an input of energy the enzyme can be occupied by ADP and  $P_i$  in one catalytic site, an ATP “trapped” in a second site, while the third site is empty. The input of energy causing rotation and hence conformational changes reduces binding and eventually allows the ATP to escape from one site, while trapping the ADP +  $P_i$  at the other. A second ATP is formed from ADP and  $P_i$ , and now a new set of substrates can enter. The three conformational states of the  $\beta$  subunit have been referred to as “open” (O), “loose” (L), and “tight” (T), and schematic representations in reviews and textbooks now use this notation (Figure 5.26).

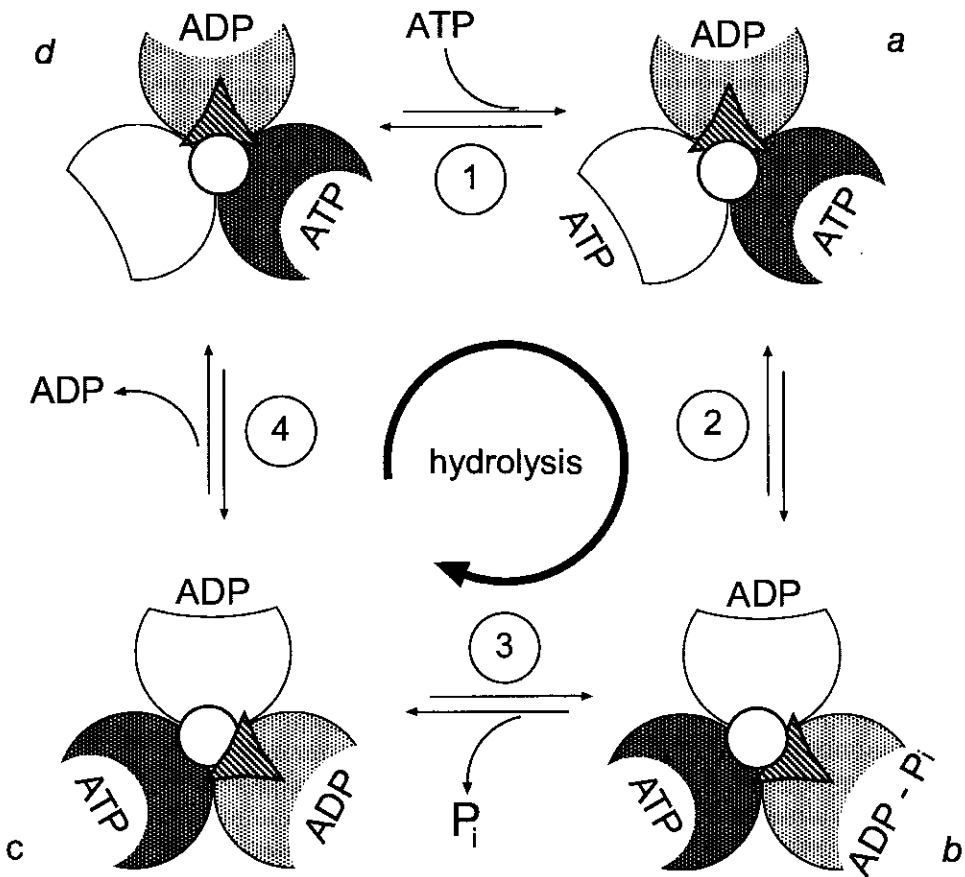


**Figure 5.26** The three-state model for the proton-driven synthesis of ATP by complex V. The threefold symmetry of the “top” (made up of  $\alpha, \beta$ , subunits) is indicated, but each of the three subdomains is in a different conformation due to the rotation of the asymmetrical inner shaft. In this model there is always at least one empty site.

Much has been learned about the mechanism by studying ATP hydrolysis instead of synthesis, which occurs with the isolated  $\alpha_3\beta_3\gamma F_1$  complex. On the other hand, pure  $\beta$  subunits do not catalyze hydrolysis at a significant rate, and even the  $\alpha_3\beta_3$  complex shows suboptimal activity and altered affinities and kinetics. Investigators in this field have made the distinction between 'unisite' catalysis and 'multisite' catalysis. Unisite catalysis involves only a single catalytic site and takes place when substrate concentrations are very low. Under such conditions individual rate constants and equilibrium constants for ATP binding/release, hydrolysis/condensation, and the release of individual products ADP and  $P_i$  can be measured, and the effects of specific mutations on the reaction can be assessed. Unisite catalysis established that the release of the products was the slow step, while the equilibrium constant for the hydrolysis/condensation reaction was near unity, indicating that multiple interactions between substrates and side chains leading to stereochemical orientation, immobilization, and polarization of ATP or ADP +  $P_i$  facilitate bond breaking or re-formation. The presence or absence of nucleotides in the noncatalytic sites has no influence on unisite catalysis and its kinetic parameters.

Multisite catalysis is, however, the dominant mode of catalysis *in vivo*, based on the estimated intracellular concentrations of substrates and the measured values for the  $K_m$  of the enzyme. Multisite catalysis is characterized by an impressive (about 5 orders of magnitude) increase in the rate of hydrolysis attributed to the highly cooperative interaction between the subunits and their catalytic sites. It is at this level that some divergence of opinion still exists about nucleotide binding to the catalytic sites. Contrary to the view presented above, the more prevalent opinion now holds that all three catalytic sites are filled with nucleotides (Mg-ATP or Mg-ADP+ $P_i$ ) during steady-state hydrolysis [162]. In other words, in the older models one site was always empty; in the new interpretation an empty site is instantly reoccupied by ADP and  $P_i$  (in the synthesis mode), and this is likely to be a rate-limiting step. The three sites are still stereochemically distinguishable, as dictated by the asymmetry of the  $\gamma$  subunit shaft, but it becomes difficult to say whether a site is occupied by ATP or by ADP +  $P_i$ . Turnover in multisite catalysis was too fast to measure occupancy and distinguish binding affinities of each site experimentally. Steady state measurements yielded a single apparent  $K_m$  value in the range of 100  $\mu M$ . These and other elegant experiments were performed in Senior's laboratory with the *E. coli* complex in which a select tyrosine residue ( $\beta 331$ ) had been replaced by tryptophan. The fluorescence signal from the three  $\beta W331$  residues is quenched when the catalytic site is occupied by a nucleotide, permitting sensitive and rapid measurements of kinetic and cooperative binding parameters [162]. The results, described and summarized in an authoritative review, lead to the revised model presented in Figure 5.27.

In the crystals used for structure determinations one site was found to be empty, one site was occupied by Mg-ADP, and the other was occupied by Mg-AMPPNP. It has been questioned whether this finding, resulting from crystallization conditions, reflects a physiologically relevant state and supports one mechanism over another [162]. The crystal structure is consistent with the asymmetry required for rotational catalysis, but since it represents a frozen structure it cannot prove rotation by itself.



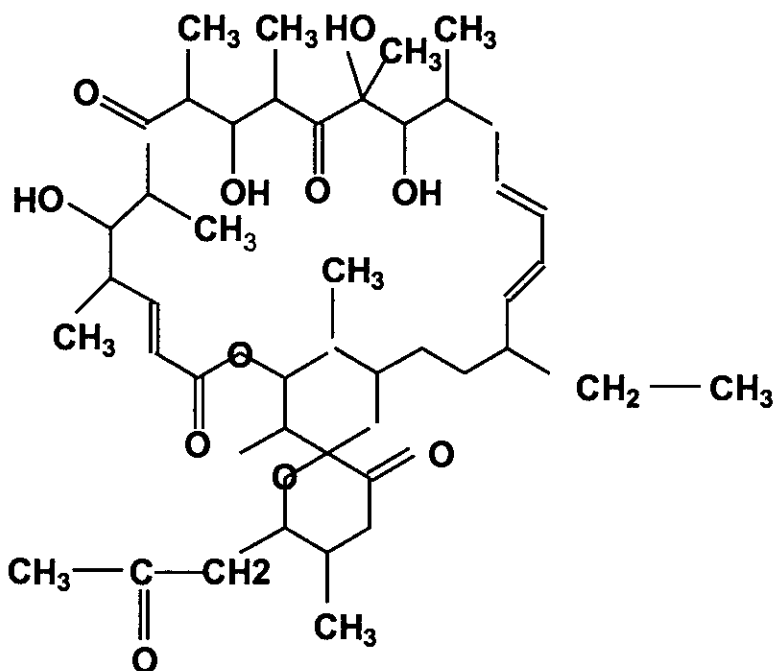
**Figure 5.27** A revised model for the mechanism of ATP hydrolysis from the analysis of multisite catalysis. In this model all three catalytic sites are occupied, except for a very transient period when the product has left and before a new substrate enters the site. The structure *D* is very short lived, and at steady state most of the molecules are in a state depicted by *C*. See text for details. (Adapted from Reference 162.)

#### 5.5.4 The $F_0$ Subcomplex and Proton Flow

Attention now must focus on the stalk and the  $F_0$  subcomplex for which high resolution crystal structures are not yet available. The problem can be posed as follows [163,174]: The  $F_0$  subcomplex provides a path (channel) for the protons. The stalk is approximately 45 Å long, and an extension penetrates the  $F_1$  subcomplex. Proton flow through the  $F_0$  channel must be transduced through the stalk into a conformational change between the  $\beta$  barrels of the  $\alpha_3\beta_3$  complex and the rotor in the center. In other words, there is likely to be no proton translocation through the  $F_1$ -ATPase itself which would couple proton translocation directly to the active site. Such a direct coupling

is probably involved in the simple proton pumps like the  $H^+$ -ATPase of the lysosome. The stalk is made up of a domain of the  $\gamma$  subunit, the  $\epsilon$  subunit, (and the OSCP in mitochondria), and it is difficult to conceive that this thin structure could accommodate an insulated proton channel, that is, represent a continuation of the channel through the  $F_0$  subcomplex. While  $\delta$  and  $\epsilon$  subunits were dispensable in the demonstration of  $F_1$ -ATPase as a rotary motor, the  $\epsilon$  subunit is likely to be necessary for the coupling between the  $F_1$  and  $F_0$  subcomplexes. The bacterial  $\epsilon$  subunit (equivalent to the mitochondrial  $\delta$  subunit) can be crosslinked via its C-terminal domain to the  $\alpha_3\beta_3$  barrel, and via its N-terminal domain to the  $\gamma$  subunit. Sequence conservation in bacterial and chloroplast  $\epsilon$  subunits has focused attention on an N-terminal domain, and studies employing site-directed mutagenesis have shown that mutations (e.g.,  $\epsilon$ H38C) affect coupling between proton flux and ATPase [163]. Cryoelectronmicroscopy has shown a major shift of the  $\epsilon$  subunit relative to the  $\alpha$ - and  $\beta$  subunits when viewed down the threefold symmetry axis upon binding of different nucleotides [163].

The mitochondrial equivalent to the bacterial  $\delta$  subunit is also known as the oligomycin sensitivity conferring protein (OSCP). Oligomycin (Figure 5.28) binds to the  $F_0$  complex and blocks proton flow. In the intact  $F_1$ - $F_0$  complex oligomycin also inhibits the ATPase, and reassembly of the  $F_1$  ATPase on the membrane is greatly enhanced by the presence of the OSCP. Thus, it has been proposed to be part of the stalk, with its precise interactions still to be clarified.



**Figure 5.28** Structure of oligomycin.



The bovine  $F_0$  subcomplex had first been prepared in Racker's laboratory by a combination of sonication and urea treatment. A more homogeneous preparation suitable for structural analysis has recently been devised by the Cambridge group of J. Walker [175]. The final complex from bovine heart contains nine different proteins (a-g, F6, A6L), confirming the stoichiometry for the mammalian mitochondrial  $F_0$  complex established independently [e.g., 157]. All of the subunits have been expressed in bacteria from cloned cDNAs, and successful re-constitution experiments making various sub-complexes as well as the entire  $F_1F_0$ -ATPase have been reported [175]. The assembly of subsets of peptides can be interpreted in a way that is similar to the interpretation of cross-linking experiments. That is to say, the affinity of two or more peptides for each other in isolation is interpreted to indicate a likely association in the complete complex.

Significant information is also available for the yeast mitochondrial  $F_1F_0$ -ATPase peptides. While there is agreement about the designation of the subunits in the  $F_1$ -ATPase ( $\alpha_3\beta_3\gamma\delta\epsilon$ ), there is unfortunately no consistent nomenclature for the remaining peptides. Thus, yeast subunits 8, 6, and 9 (encoded by the mitochondrial genome) appear to correspond to the a, b, and c type subunits of bacterial, chloroplast, and mammalian  $F_0$  [176]. The yeast peptides have been characterized from purified complex V, but a genetic analysis of respiration-deficient yeast mutants has contributed significantly to the identification of the complex V structural genes. Additional gene products were found to be necessary for the assembly of the complex, but were not present in the purified complex [176].

From comparisons with the simpler complex in bacteria one can conclude that three essential peptides make up the proton-translocating hydrophobic core [174]. Subunit a (271 residues in bacteria) has multiple transmembrane regions, most likely an even number (4–8). This places both the N-terminal and the C-terminal on the same side of the membrane. With the help of fusion proteins the ends have been placed on the outside (periplasmic side) of the bacterial membrane [174]. It is not required for the binding of the  $F_1$ . The b subunit may be present as a dimer. In bacteria, it has been deduced from the amino acid sequence that subunit b has a single transmembrane segment near the N-terminal, with the remaining domain forming a predominantly  $\alpha$ -helical, highly charged secondary structure. This hydrophilic domain was thought to form part of the stalk structure by interacting with the  $\gamma$  and  $\delta$  subunits of  $F_1$  described above. In its absence, the assembly of the  $F_1$  with the  $F_0$  is severely perturbed [174]. However, a revised model places the two b subunits on the outside of the  $\alpha/\beta$  complex where it acts as the stator (Figure 5.23B). The c subunit is present in multiple copies (9–12) compared to the a subunit. Within its short sequence (79 residues in bacteria) two transmembrane  $\alpha$  helices are predicted, flanking a polar loop region which is involved in the binding of  $F_1$  to  $F_0$ . The polar loop also appears to have a special role in coupling ATP synthesis to proton translocation. Curiously, a Q42E mutant c-peptide allowed normal assembly of the  $F_1F_0$ -ATPase, but ATP hydrolysis was uncoupled from proton translocation (the bacterial enzyme is fully reversible depending on physiological conditions) [174]. The well-known inhibition of the  $F_1F_0$  ATPase by DCCD is due to a unique reaction of Asp61 (D61) with dicyclohexylcarbodiimide (DCCD). Even if only a single c subunit is deriva-

tized by DCCD, the ATPase activity of the whole complex is abolished. Further studies of the isolated subunit c in a chloroform-methanol-water mixture are encouraged by experiments suggesting that the isolated protein retains features of the native protein, including the reactivity of D61 with DCCD, and the inhibition of this reaction by the I28T mutation. Experiments of this type and mutational analysis at this Asp residue have been used to argue that the carboxyl group of D61 is a participant in proton translocation. At this time it appears to be the best candidate [174]. The interaction of protons with the buried D61 was postulated to trigger conformational changes, which also affect the polar hairpin loops of the c subunit. Confirmation of this concept has been obtained from NMR studies. Similarly, a mutational analysis of the bacterial a subunit has identified the R210 residue as an absolutely essential residue for proton translocation. Thus, the single a subunit and 9–12 c subunits form a path for proton flux across the membrane.

The 1:2(?) : 9–12 (a:b:c) stoichiometry in the  $F_0$  presents another significant puzzle. Since a and b can be crosslinked, it has been proposed that they form a core around which the multiple c subunits are arranged within the plane of the membrane. This would place different c subunits into sterically different positions. An alternative and currently favored model (Figure 5.23B) places the a and b subunits of  $F_0$  on the outside of the c oligomer [160,162,166], where the b dimer specifically is postulated to play the role of a stator (in conjunction with the bacterial  $\delta$  subunit). In this view the  $b_2$  dimer is not involved in forming the proton channel. However, placing the a subunit on the outside clearly leads to a highly asymmetric structure. As proposed by several authors [see Reference 162 for references], the a subunit is envisaged to rotate around the core c oligomer. With reference to Figure 5.23B, the revised model of the Capaldi group [160,161], one is compelled to ask the following question: What is rotating and what is static within the  $F_1$ - $F_0$  complex, and which part of the complex rotates relative to a fixed point in the plane of the membrane? Only the motion of  $\gamma$  relative to the  $\alpha\beta$  complex has been firmly established so far. Is the rotation of the central shaft ( $\gamma$ ,  $\epsilon$ ) coupled to the rotation of the c oligomer of the  $F_0$  subcomplex within the lipid bilayer? Or does the outer shaft (made up of a,  $b_2$ , and the bacterial  $\delta$  subunits) also rotate around the  $\alpha\beta$  hexamer, presumably in synchrony with the inner shaft. The mechanical and hydrodynamic aspects of such rotations remain to be explored. It would obviously be simpler to have at least the a subunit in the center of the c oligomer and allow it to rotate together with the  $\gamma/\epsilon$  shaft.

Proton flow in such a molecular motor must produce a torque. A priori inertial and frictional forces have to be considered, but inertial forces are estimated to be negligible. Proton transfer must be associated with a rotation or displacement of an a subunit relative to a c subunit. The model must also include the experimental fact that several protons and several c subunits are involved in a single move (see below). Junge and his colleagues [166] made some detailed suggestions in a recent review, but also state that “the concept of an ‘electrochemical-mechanical-chemical nanoengine remains a challenge for critical examination.”

It can be expected that the pure  $F_0$  complex from bacteria or bovine mitochondria will be structurally characterized in the not too distant future. Fitting it together with the  $F_1$  will then be the next challenge, and the questions raised above will be resolved.

Finally, there still appears to be some disagreement about the number of protons required per ATP synthesized [162]. Opinions on the  $H^+$ /ATP stoichiometry sway between the values of 3 and 4. A resolution of this conflict is important not only for the bookkeeping on the energetics of oxidative phosphorylation. It would determine the efficiency of converting proton flow down the electrochemical and concentration gradient into ATP synthesis. Of greater interest, however, is the number of protons per 120-degree turn of the rotor, and the implications of this number for the detailed mechanism of proton translocation through the  $F_0$  subcomplex. For example, a value of 4 might imply that four c subunits are protonated per ATP synthesized, and the likely total number of c subunits in the oligomer is 12; a value of 3 might suggest a total of 9 c subunits/ $F_0$ .

It is indeed remarkable what has been learned already about this most important motor for life on earth. When will we be satisfied to say that we know everything?

## 5.6 CONTROL OF RESPIRATION AND OXIDATIVE PHOSPHORYLATION

### 5.6.1 General Considerations

It is quite apparent that the energy demands of a given cell or tissue will vary over time, and the need for regulatory mechanisms becomes obvious. A distinction to be made at the beginning of this section is to separate mechanisms involved in long-term control, in the course of development and tissue differentiation, from short-term control mechanisms operating on a time scale of seconds to hours. A prominent example is muscle, but other tissues and organs in mammals and other organisms may also exhibit fluctuations in respiration and oxidative phosphorylation in response to regulatory signals from hormones, growth factors, or the nervous system. A subtle but very important aspect of this problem is the balancing between the capture of the free energy from combustion in the form of ATP, and the release of some of that energy in the form of heat. This is of course the basic mechanism for maintaining the body temperature in warm-blooded animals, and specific tissues and mechanisms have evolved for this task.

The long-term control in differentiating tissues, or in the adaptation of a microorganism such as yeast to changing environmental conditions, is likely to be based on the control of gene expression and the rate of biogenesis of mitochondria or the components of the inner membrane. In Chapter 3 a discussion of the morphology of mitochondria and of the density and shape of cristae has made reference to the likely explanation that the density of cristae reflects the capacity for respiration of the particular cells examined.

The present discussion will focus on short-term control. A highly readable and thoughtful review of this subject with many primary references has been written by Brown [177]. It concludes with the statement that “there is no simple answer to the question ‘what controls respiration?’”. The question becomes increasingly more complex as one considers an isolated mitochondrion, a uniform cell population in the con-

trolled environment of the culture medium, or an organ. The answer is likely to vary also depending on the source of the mitochondria. Even more variations will have to be considered when one compares mitochondria from different organisms. One may also have to make a distinction between studies with isolated mitochondria where extremes of conditions can be explored, and *in vivo* situations where the parameters cannot be so well controlled or are not even precisely known. In the following discussion, therefore, the emphasis will be on the basic principles which will have to be taken into consideration when the question of the regulation of mitochondrial activity is raised.

A formal analysis of the problem that has led to important insights and conclusions is based on the theoretical framework now called Metabolic Control Analysis [178,179]. The "Control of Flux" was a landmark paper by Kacser and Burns first published in 1973, and as a recent updated and annotated reprint by Kacser [178]. It begins with the emphatic statement that "flux is a systemic property and questions of its control cannot be answered by looking at one step in isolation—or even each step in isolation," and it ultimately challenges our familiar and simplifying concept of a "rate controlling enzyme," or of a "bottleneck in the pathway." In its explicit formulation it constitutes a highly mathematical treatment seeking to express how sensitive a flux in a pathway is to an enzyme's concentration or activity. A flux control coefficient is introduced as a quantitative measure of the system's sensitivity to modulations of one of its components (enzyme activity/concentration), and it is a measure of the importance of this enzyme for control of flux through the pathway, regardless of whether and how this enzyme is controllable. A coefficient of 1.0 would indicate that an enzyme is controlling the pathway, and in practice that a 1% increase in enzyme concentration/activity would lead to a 1% increase in flux. Such an enzyme would constitute the typical "bottleneck" in a pathway. Conversely, a coefficient of 0 would indicate that the enzyme is in excess and changing its concentration fractionally would not lead to a significant change in the flux. It appears that the control coefficient for many enzymes is small, hence heterozygous individuals are normal, and mutations inactivating one allele are classified as recessive. Post-transcriptional mechanisms may influence such simple considerations based on gene dose. The formal treatment also introduces elasticity coefficients for each enzyme, formerly known as the controllability coefficients, which roughly define the sensitivity of an enzyme to all the metabolites and effectors which interact with it. The reader is referred to the original literature for a formal discussion of the terminology and mathematical treatment.

While the treatment is highly theoretical and lends itself to explicit computer simulations, it is also highly suitable for the interpretation of experimental data from well-known pathways such as glycolysis and oxidative phosphorylation. Input substrate concentrations can be varied, specific inhibitors can be used to modulate the activity of individual enzymes in the pathway, and more recently it has become possible to manipulate individual enzyme levels by genetic methodologies (e.g., in yeast). On the other hand, the best computer model is useless if it is too simple to reflect the reality inside the cell.

Applications of metabolic control analysis to oxidative phosphorylation have been numerous over the years, and experimental systems have included isolated mito-

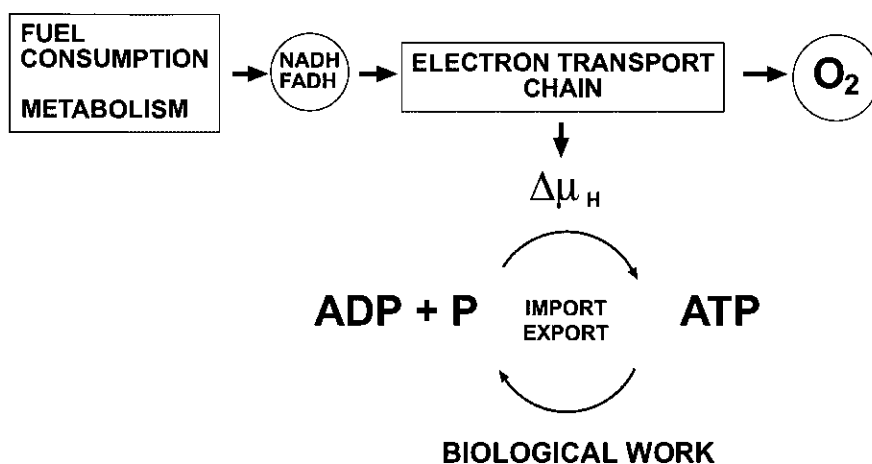
chondria, intact cells, perfused tissues and whole living organisms. Recent examples by Brand and his colleagues [180] describe the metabolic control analysis of the effects of cadmium on oxidative phosphorylation in mitochondria isolated from potato tubers, and the control of oxidative phosphorylation in liver mitochondria and hepatocytes [181]. Before discussing the formalism used by this group and the conclusions derived from this “top-down approach” (i.e., “starting from the top and analysing the whole system”), a more conventional set of considerations will be presented.

### 5.6.1.1 The Role of Substrates

It is useful to start with a broad view of the overall problem in which the major reactions are grouped as follows (Figure 5.29):

With this perspective one can ask a few simple questions which may or may not be easy to answer depending on the experimental system chosen. For example, some definite answers may be obtainable with isolated mitochondria, but the *in vivo* conditions may be less well known.

Based on some early work by H.A. Lardy and B. Chance with isolated mitochondria the idea evolved that the major controlling reaction is the rate of consumption of ATP and hence the availability of ADP (and  $P_i$ ). In other words, when oxidizable substrates (fuels) were supplied, the rate of oxygen consumption depended mainly on the supply of  $ADP + P_i$ . Failure to provide a substrate to complex V was assumed to back up the electron transport chain by mass action, and because electron transport and ATP synthesis were tightly coupled. If the  $\Delta\mu_H$  could not be dissipated by ATP synthases, further proton translocation and electron transport was inhibited upstream from the cytochrome oxidase. For example, the antibiotic oligomycin has been known for a long time to inhibit respiration by binding specifically to a peptide of the  $F_0F_1$  ATP synthase and inhibiting ATP synthesis (see Section 5.5). Mitochondria supplied



**Figure 5.29** Schematic view of the major reactions in the consideration of the control of respiration and oxidative phosphorylation.

with a fuel (e.g.,  $\beta$ -hydroxybutyrate) and an excess of ADP + P<sub>i</sub> are said to be in “state 3” and maximum respiration (oxygen consumption) is observed. In the absence of ADP the mitochondria are said to be in “state 4,” and oxygen consumption can be an order of magnitude lower. Under such conditions the rate of respiration is controlled by the proton leakage through other porters, channels, -often ill-defined. It is clear that meaningful experiments require intact mitochondria, and mitochondria which have been stored for a while or have been frozen exhibit increased proton leakage and hence less and less “tight coupling.” One way to increase respiration in the absence of ADP is to abolish the proton gradient. This can be accomplished in several ways. The addition of nigericin (a K<sup>+</sup>/H<sup>+</sup> antiporter) can collapse the  $\Delta$ pH without destroying the membrane potential  $\Delta\Psi$ . Alternatively, the classical uncoupler dinitrophenol can collapse both  $\Delta$ pH and most of the  $\Delta\Psi$  by providing a path for protons across the lipid bilayer. The action of naturally occurring proteins in brown adipose tissue which cause proton leaks and thus are uncouplers will be elaborated below in the discussion of thermoregulation.

ATP consumption occurs mostly in the cytosol. The adenine nucleotide carrier (ANC), also known as the ATP/ADP antiport system, therefore can become a potential regulatory site. It controls the flux of ATP out of the mitochondria, of ADP into mitochondria, and is electrogenic, hence sensitive to the membrane potential. Influx of ADP also transports a proton into the matrix and decreases  $\Delta$ pH, thus stimulating electron transport. The phosphate carrier is electroneutral, and at least in liver its capacity far exceeds the requirements for ATP synthesis. It is not likely to be a regulator of OXPHOS.

Typically the ratio of [ATP/ADP] *in vivo* is very high (~100:1 in the cytosol; is it different in the matrix?). As a result, changes in relative levels of ATP are small compared to relative changes in ADP. In addition, the ATP concentration may be buffered by the pool of creatine phosphate (depending on the tissue). Controversy has centered around the question whether the relevant parameter is the change in [ATP], the change in [ADP], a change in the [ATP]/[ADP] ratio, or even a combination including [P<sub>i</sub>]. A general consensus appears to favor the view that the control of respiration by ATP consumption is mainly due to changes in [ADP] inside the mitochondria.

One of the more explicit treatments of energy metabolism and its relationship to ATP utilizations was developed by Atkinson [182], who proposed that the key parameter was the energy charge defined by

$$\frac{[\text{ATP}] + 0.5[\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]}$$

a measure of the fraction of the total adenylate pool which is activated relative to AMP. The treatment includes allosteric effects on cytosolic enzymes (glycolysis) as well as on mitochondrial dehydrogenases, and ATP utilizing pathways. It represents a major attempt to draw generalizations about metabolic control, and to present a theoretical framework to confront an overwhelming problem.

Other formal thermodynamic treatments have led to a “near equilibrium hypothesis” [183,184]. Most of the steps in the above scheme were considered to operate near

equilibrium, with the exception of cytochrome oxidase. Respiration and ATP synthesis became functions of 1) the NADH/NAD ratio, and 2) the  $[ADP][Pi]/[ATP]$  ratio, and further “tuning” of the cytochrome oxidase by effectors such as pH or  $pO_2$ .

The  $K_m$  of isolated cytochrome oxidase (absence of  $\Delta\mu_H$ ) for oxygen has been estimated to be in the micromolar range, and an apparent  $K_m$  for oxygen in isolated mitochondria has been measured to be in the same range, or even significantly lower (differing in tightly coupled mitochondria from uncoupled mitochondria). It is believed that most cells have oxygen concentrations far above these values, although steep gradients within tissues may exist or be created temporarily. Most likely, therefore, oxygen is not a rate limiting substrate in mammalian tissues. Different considerations may apply when anoxia is encountered as a result of injuries. In several tissues (brain, liver, kidney) respiration has been measured under conditions when the oxygen supply was far below the physiological levels. Initial rates of oxygen consumption were normal, but a subsequent decline was observed, probably due to secondary effects.

Looking at the beginning of the scheme, one may ask whether the supply of NADH is ever a rate limiting factor. With isolated mitochondria in vitro such conditions can be created easily, but under physiological conditions in vivo it is less certain whether NADH is in short supply. (This statement may not apply to microorganisms such as yeasts, molds, and fungi, where environmental conditions are likely to include periods of starvation). The electron transport chain is limited by the intrinsic rate of electron transfer between the various centers, and it cannot be “pushed” by supplying excess NADH. At any rate, this substrate cannot be supplied directly. Instead, substrates such as  $\beta$ -hydroxybutyrate or succinate are added to isolated mitochondria; cells or tissues can be given glucose. As will be discussed below, there are elaborate feedback mechanisms which can shut down the Krebs cycle when NADH levels rise above a threshold.

#### 5.6.1.2 Control of Enzyme Activity

The parameters discussed up till now have been the immediate substrates of oxidative phosphorylation: NADH, ADP,  $P_i$ , protons, and oxygen. So far, their influence on respiration and oxidative phosphorylation has been viewed as a mass-action effect in a system of constant cycling. NADH is resupplied by many diverse reactions which are individually regulated at various levels, and the ADP supply appears to depend on the biological work performed by the cell. Protons exert an effect because they are substrates of vectorial reactions operating near equilibrium. One can now ask whether the specific activity of the individual complexes can be modulated by various potential effectors:  $Ca^{2+}$  ions, free fatty acids, hormones, adenine nucleotides, and specific proteins have been investigated.

Cytoplasmic calcium levels are controlled by extracellular signals such as hormones and growth factors, and most prominently by electrical signals in the case of muscle. Mitochondria have an electrogenic uniporter for import and a  $Ca^{2+}/Na^+$  exchanger for efflux [185,186] which keep the matrix concentration of  $Ca^{2+}$  in the range of 100 nM to 1  $\mu$ M. Increases in free  $Ca^{2+}$  are thought to stimulate various dehydrogenases in the Krebs cycle; pyruvate dehydrogenase may be stimulated indirectly by

$\text{Ca}^{2+}$  by a conversion to the unphosphorylated, more active form through an activation of a phosphatase. Therefore, the NADH supply is increased, but not necessarily the rate of respiration. The effect of cytosolic  $\text{Ca}^{2+}$  on muscle activity is well known, and muscle contraction utilizes ATP. There are some observations with liver mitochondria suggesting that  $\text{Ca}^{2+}$  may be involved in changes in mitochondrial volumes following a hormonal signal [186], and this effect in turn is believed to be responsible for increased electron flow. However, alternative explanations have been offered [177].

The  $[\text{ATP}]/[\text{ADP}]$  ratio may have an influence on the relative activities of kinase/phosphatase couples controlling specific enzymes such as pyruvate dehydrogenase (see above), or either of these nucleotides may be an effector in the control of Krebs cycle enzymes. For example, ADP has been shown to be an allosteric activator of isocitrate dehydrogenase by decreasing the  $K_m$  for isocitrate. Again, the NADH supply is controlled and elevated at higher ADP concentrations. Both ATP and ADP have been shown to be allosteric effectors of cytochrome oxidase [187].

A special mechanism influencing the  $[\text{ATP}]/[\text{ADP}]$  ratio is the activity of so-called futile cycles. Such cycles require a substrate (e.g., fructose 6-phosphate), a specific kinase and a specific phosphatase which may be independently regulated. By combining the two activities a net ATPase mechanism is generated, and ATP wastage can be regulated. Increasing the rate of such a futile cycle can lead to increases in the rate of respiration.

Because cytochrome oxidase does not operate on a reaction near equilibrium, it has been considered as a bottleneck and rate-limiting enzyme in electron transport. An intriguing observation is that complex IV is the only complex with tissue-specific subunits (isozymes) [98,188]. Subunits VIa, VIIa, and VIII have been characterized to have a liver (L) form and a heart (H) form. With reconstituted cytochrome oxidase from either bovine heart or liver the laboratory of Kadenbach has demonstrated that ADP interacts with and stimulates the heart enzyme but not the liver enzyme [189]. The effectiveness of intra-liposomal ADP on reconstituted COX was interpreted to indicate an interaction with the matrix domain of the enzyme, and specifically with the 17 N-terminal amino acids of subunit VIa-H. This interpretation was strengthened by the demonstration that a monoclonal antibody directed against the VIa-H but not VIa-L form can also enhance the activity of the enzyme, presumably by inducing a conformational change in the heart enzyme [187].

An even more general speculation has been proposed by Kadenbach's group: all of the nuclear-encoded subunits of complex IV may serve as targets for allosteric effectors, with no role in the catalytic function of the enzyme. Such a proposal is based on the comparison of prokaryotic cytochrome oxidases with eukaryotic cytochrome oxidases. In *Paracoccus denitrificans* two subunits are sufficient for activity, while in eukaryotes a variable number of additional subunits (5 in *Dictyostelium*, 6 in yeast, 10 in mammals) are found [190].

A very recent observation has been published leading to the radical claim that the  $\text{H}^+/\text{e}^-$  stoichiometry of cytochrome oxidase from bovine heart is regulated by the intramitochondrial  $[\text{ATP}]/[\text{ADP}]$  ratios [191]. If verified, it would constitute a challenge to one of the basic postulates of vectorial metabolism as defined by Mitchell. In this case, an effector would regulate not only the activity of an enzyme, but would also



control the stoichiometry of the reaction (a “slipping pump”). To reemphasize: a P/O ratio which is not an integer can be rationalized by the chemiosmotic hypothesis, but a variable  $H^+/e^-$  ratio for a given complex, and the reaction carried out by this complex, would be quite unorthodox.

### 5.6.1.3 Flux Control

Returning to the formalism of metabolic control analysis by Brand and colleagues [180,181], the simplest version considers three blocks of reactions: substrate oxidation (succinate oxidation), ATP turnover (using exogenous hexokinase as ATP consumer), and proton leakage, all connected by the common intermediate  $\Delta p$ , the proton-motive force, measured as  $\Delta\Psi$  by the distribution (and fluorescence) of methyltriphenylphosphonium. Titrations with an inhibitor of substrate oxidation (malonate), with an inhibitor of the  $F_0F_1$  ATPase (oligomycin), and in the presence of various uncoupler concentrations yield experimental parameters, which can be used to calculate flux control coefficients applied to respiration rates under variable conditions. The analysis confirms earlier conclusions that control over respiration rate ( $O_2$  consumption) is shared between the three blocks of reactions: there is no single, clearly outstanding rate limiting step. When respiration is maximum, ATP turnover and substrate oxidation are about equally important (control coefficients  $\sim 0.5$ ), while proton leakage is almost insignificant. When the respiration rate is 60% of maximum, ATP turnover dominates (control coefficient:  $\sim 0.7$ ), while substrate oxidation and proton leakage are equally but less influential. Conditions must be specified to talk about control of OXPHOS.

The authors also discuss their results in terms of coupling efficiency and P/O ratios using the same data set. Not surprisingly, it is strongly negatively controlled by proton leakage, and strongly positively controlled by ATP turnover, while it is insensitive to substrate oxidation. In other words, effectors acting only on substrate oxidation will not affect the coupling efficiency, but the efficiency can be increased by increased ATP turnover.

While the simple model introduced above is a powerful model to investigate the actions of hormones and of other effectors on OXPHOS in a given tissue (“Once you get the hang of it, both the theory and experimental applications are simple” [181]), the simplest model treats the entire electron transport chain as “one enzyme,” and hence is not concerned with control coefficients of individual complexes (and mobile carriers such as cytochrome c). However, the latter problem becomes of interest in the understanding of mitochondrial diseases, when the activity of individual complexes may be affected by specific mitochondrial mutations (see Chapter 7). Korzenievski and his colleagues [192] have elaborated the model considerably by decomposing the substrate oxidation into three reactions involving complexes I, III, and IV. Theoretical calculations for muscle mitochondria respiring on pyruvate were made, and control coefficients were calculated for each of the complexes (I, III, and IV), and separately for substrate dehydrogenation (pyruvate to generate NADH), complex V, the ATP/ADP carrier, the phosphate carrier, proton leakage, and ATP turnover (hexokinase in the presence of glucose). The highest flux control coefficient was calculated for complex III in state 3 mitochondria: 0.26. In other words, control is again distrib-

uted over many reactions. Under physiological conditions, (state 3.5 mitochondria), control coefficients are generally less than 0.1 for the complexes I, III, IV, and V, while the coefficient for ATP turnover was 0.8. Availability of ADP thus controls the respiration rate. More significantly, the low control coefficient for complex IV (0.07 in state 3) can explain why the respiration rate is almost unaffected when the activity of complex IV is titrated with an inhibitor to the level of  $\sim 80\%$  inhibition; at  $>80\%$  inhibition respiration declined sharply. In other words, cytochrome oxidase activity is considerably in excess of normal requirements. Establishing a threshold of inhibition at which the enzyme becomes limiting may be highly relevant for the understanding of the physiological consequences of heteroplasmy and mitochondrial mutations. It is equally important to make such measurements with tissues, cells, or isolated mitochondria in which the true physiological parameters are maintained.

### 5.6.2 The Uncoupling Proteins in Warm-Blooded Animals

The proton gradient established by electron transport can be dissipated in two ways: ATP synthesis through complex V, or proton leakage through the inner membrane without the production of ATP to be used for other chemical and biological work. By mechanisms described above, respiration may be controlled by ATP/ADP levels and turnover in tightly coupled mitochondria. As a first approximation one can describe tightly coupled mitochondria as mitochondria in which proton leakage outside of complex V is minimized. The combustion of carbon compounds to  $\text{CO}_2$  is also accompanied by the release of a fixed amount of heat, the inevitable by-product of chemical reactions occurring at finite rates. The free-energy change associated with the conversion of glucose to  $\text{CO}_2$  and water is distributed between the heat released and the useful free energy temporarily stored in the ATP pool. Additional heat will be released from the hydrolysis of ATP. Thus, a mechanism is in principle provided to increase our body temperature above ambient temperatures.

It has been recognized for some time that a constant body temperature in warm-blooded animals is maintained not only by conducting excess heat away (perspiration, etc.), but that at low temperatures additional heat must be generated. Two mechanisms have been identified: 1) thermogenesis by shivering in effect increases ATP turnover and respiration in muscles, hence the production of heat; 2) nonshivering thermogenesis was discovered in newborns and hibernating animals, and the primary role of brown adipose tissue was established. Brown adipocytes were subsequently characterized to possess a unique mechanism for increasing respiration without ATP synthesis (uncoupling). A specific protein was found to be induced in adipose cells which could transfer protons across the inner mitochondrial membrane, i.e. effectively short circuit the proton circuit, resulting in increased respiration and heat production. The role of this uncoupling protein (UCP-1, or thermogenin) in brown fat in relation to development, cold adaptation, diet-induced thermogenesis, and genetic obesity has been studied extensively, and a number of authoritative reviews have been written [e.g., 193,194]. On the other hand, it also became apparent that adult animals and humans do not have significant amounts of brown adipose tissue, and hence the problem of thermoregulation in adult mammals could not be easily explained with the

help of UCP-1. It was argued, that in adults thermoregulation may occur in other tissues and cell types, perhaps by means of a similar mechanism involving a related protein. Such a protein, called UCP-2, was recently discovered by a genetic approach and found to be related mechanistically and in sequence to UCP-1 [195]. In contrast to UCP-1, it is expressed in a wide variety of tissues. By regulating the level of these proteins by any one of a number of pathways which can include hormonal signals, transcriptional control, translational control and control of stability, it therefore becomes possible to fine tune the proton leakage in mitochondria, and hence uncouple respiration and heat production from ATP synthesis. Further considerations suggest that these proteins, and particularly UCP-2, may play a role in diabetes (glucose metabolism), and obesity [195].

The study of UCP-2 is less well advanced because of its recent discovery, but it is likely that insights gained from the study of UCP-1 will be directly applicable to UCP-2. A major distinction is likely to be in the control of their expression and tissue distribution.

The history, arguments, and experimental evidence leading to the identification of UCP-1 in brown adipose tissue have been expertly reviewed by Nicholls and Locke [193]. The presence of an uncoupler was suspected by the observation that brown adipose cells had insufficient ATPase activity (total) to account for the observed oxygen consumption coupled to ATP turnover. Free fatty acids were first thought to be involved in the uncoupling mechanism, but later a "nucleotide-induced recoupling" was added and distinguished from the "fatty acid-induced uncoupling." The binding and activity of nucleotides was a defining characteristic which led to the first isolation of UCP-1 from mitochondria in 1978. A high-affinity, saturable binding site for [ $^3\text{H}$ ]-GDP on the outer surface of the inner membrane could be demonstrated to be identical to a component in the inner membrane responsible for short circuiting the membrane for protons, i.e. with activity as a proton uniporter.

A small protein of molecular mass 33 kDa was found to be present exclusively in brown adipose tissue from a variety of mammals, accounting for 6–14% of the inner membrane protein. Following the discovery by Nicholls and his group, significant contributions to the characterization of the protein and its activity have been made by Klingenberg and coworkers [see References 196 and 197 for a recent publication with references to past work]. Another detailed discussion can be found in the review by Nedergard and Cannon [198]. It was shown that  $\text{H}^+$  transport requires free fatty acids as obligatory cofactors, and is inhibited by adenosine and guanine di- or triphosphates. Nucleotide binding affinity decreases with pH. The pH sensor for nucleotide binding has been identified, and incorporated into a model for the orientation of the UCP transmembrane helices in the membrane [197]. The protein shares some homology with the ADP/ATP transporter (antiporter) and has therefore been included in the mitochondrial carrier family [196]. Both proteins bind nucleotides, but in one case the nucleotide is a substrate capable of binding to either side, while in the other it is a regulator binding only the cytosolic side. They both form functional dimers in the inner membrane, with a single binding site per dimer. The entire carrier family includes characteristically short peptides with three repeat structures of approximately 100 residues, each repeat containing two transmembrane

helices. A monomer therefore has six transmembrane helices; the N-terminal and C-terminal domains extend into the intermembrane space. The connecting loops on the matrix side are made up of around 40 highly polar residues. An interesting contrast between these two carriers has also been emphasized: UCP-1 transports the smallest ions  $H^+$  (or  $OH^-$ ) unidirectionally, while the ATP/ADP transporter exchanges very large solutes. A further discussion of the ATP/ADP transporter can be found in Section 5.6.4.1.

Uncoupling by UCP-1 can therefore be regulated on at least two levels. The carrier itself is sensitive to the proton gradient and the pH in the intermembrane space as well as to nucleotide concentrations and fatty acids, and such mechanisms lend themselves to rapid short term control. A plausible signaling pathway has norepinephrine bind to the  $\beta$  receptor, causing a rise in cAMP, activation of a protein kinase which controls lipolysis (by a lipase), and eventually accumulation of free fatty acids. Nucleotide inhibition is released, and oxidation of acyl carnitine and acyl-CoA can proceed at an increased rate [193]. There is, however, also a long term control of thermogenic capacity related to the dietary status of an animal such as a rat, or its adaptation to prolonged exposure to cold. The correlation of thermogenic capacity with the levels of the UCP-1, and  $\beta$ -adrenergic stimulation of brown adipocyte metabolism and respiration have been subjects which have received much attention. Nicholls and Locke [193] present the topic from a more physiological perspective. In the meantime, the cloning of the UCP-1 gene has permitted an analysis of its promoter and the control of its expression. An up-to-date review [194] lists thyroid hormone and retinoic acid response elements as having a direct influence, while catecholamines act via a cAMP pathway. Insulin, glucocorticoids, and IGF-I may modulate gene expression via more indirect pathways, and CCAAT enhancer binding proteins and peroxisome proliferation activator receptor  $\gamma 2$  have also been implicated in control. UCP-1 mRNA appears to have a short half-life, permitting rapid fluctuations in UCP-1 mRNA steady state levels depending on the ambient temperature, food intake, and hormone release.

One can expect similar detailed analyses of the UCP-2 promotor and its transcriptional activators or repressors. Not only our understanding of thermoregulation will be increased substantially, but the relationship to genetic obesity and cold intolerance will be clarified. The promise of therapeutic intervention and even gene therapy is on the horizon.

The small size and simplicity of the UCP-1 structure have invited speculations and experiments about the mechanism of proton translocation. An even more powerful approach to analyzing its structure in relation to function is the successful expression of the mammalian UCP-1 in yeast, and the demonstration that it retains its properties: proton and chloride transport, high affinity binding of nucleotides, and the dependence of conductance on nucleotides and fatty acids [199]. Site-directed mutagenesis can now be employed to investigate systematically the functional role of various amino acid side chains. Chemical modifications had already implicated several cysteine residues in activity, and models based on dithiol-disulfide interconversions had been proposed. Surprisingly, the replacement of all seven cysteine residues by serine was subsequently found to have no effect on acidity or regulatory characteristics of the UCP-1 in yeast [199].

### 5.6.3 Uncoupling in Other Organisms

#### 5.6.3.1 *In Saccharomyces cerevisiae*

The growth of yeast on a variety of carbon sources (glucose, glycerol, acetate, ethanol) requires a large number of adjustments to optimize growth rates under conditions which may constitute a carbon limitation or an energy limitation. Theoretical considerations of the need to maintain redox balance, and the observed discrepancy between ATP requirements for biomass formation with expected yields of ATP production from substrate oxidation has suggested that oxidative processes uncoupled from ATP production must be operative [200]. Among the mechanisms considered have been futile cycles (see above), and increased proton leakage. Prieto and colleagues [200–202] in a series of papers have provided details on the activation of a proton-conductance pathway by ATP. At low  $[ATP]/[Pi]$  ratios binding of ATP to cytochrome oxidase alters its kinetic properties, but at higher ratios ATP binding to the outer side of the inner membrane can increase membrane permeability and cause the collapse of  $\Delta pH$  or  $\Delta \Psi$  [201].

Understanding the properties of the endogenous ATP-induced proton channel in yeast mitochondria was an important prerequisite for the successful expression and study of the mammalian UCP-1 in yeast [199]. In the presence of phosphate, the two uncouplers could be clearly distinguished. One can also note that the identity of the newly isolated UCP-2 gene product was confirmed by its expression in yeast, where its activity as an uncoupler was even more pronounced than that of UCP-1 [195].

#### 5.6.3.2 *In Plants*

A previous section has emphasized that plant mitochondria have all of the components found in animal mitochondria, making oxidative phosphorylation in plant mitochondria a very similar process. At the same time, plant mitochondria have several unique features, most notably a cyanide-insensitive electron pathway not coupled to phosphorylation. Therefore, there appears to be no need for a mechanism requiring a specific uncoupling protein, when oxidative reactions have to be speeded up at certain developmental stages, in specific tissues, or under defined physiological conditions. However, a first report on the cloning of a potential uncoupling protein from a potato flower has recently appeared [203]. It is 44% and 47% identical to the human UCP1 and UCP2, respectively, and is induced after exposure to cold temperatures. Although there is still uncertainty and controversy about the physiological significance of cyanide-resistant respiration (see above), there is one class of examples where the operation of this uncoupled process for the apparently sole purpose of heat production is understood in physiological and functional terms. In the male reproductive structure of cycads, and in the flowers or inflorescences of species belonging to the families Annonaceae, Araceae, Aristolochiaceae, Cyclanthaceae, and Nymphaeaceae an accumulated supply of starch is metabolized (glycolysis) and oxidized by the alternate pathway in at times a matter of hours. The temperature in the structures involved can reach 15°C above the ambient temperature. This thermogenesis is useful in at least two ways. In some instances it simply permits the plant to flower under climatically adverse conditions, i.e. early in a season; in other examples it has been

clearly established that the elevated temperatures help to volatilize insect attractants (e.g., the putrescent odor of skunk cabbage) to gain the attention of pollinating insects [104,106,204].

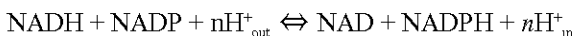
### 5.6.4 Transport of Small Solutes Into and Out of Mitochondria

Although numerous references to the transport of various ions across the mitochondrial inner membrane have been made in the sections above, it seems worthwhile to focus on this problem once more as a distinct problem. The following ions and small solutes feature prominently in discussions of mitochondrial functions:  $H^+$ ,  $P_i$ , ADP/ATP,  $Fe^{2+}$ ,  $Cu^{+1}$ ,  $Zn^{2+}$ ,  $Ca^{2+}$ ,  $NAD^+/NADH$ , and a large variety of small organic compounds which are metabolites associated with biochemical reactions. The first three have received a maximum of attention because of their role in oxidative phosphorylation and the Mitchell hypothesis.  $Fe^{2+}$ ,  $Cu^{+1}$ , and  $Zn^{2+}$  ions have been encountered as constituents in the electron transport chain (cytochromes, iron-sulfur centers, complex IV) and in metalloproteases. Mitochondria are prominently mentioned in the context of the regulation of intracellular calcium; they may constitute a reservoir or buffer, and/or their activities may themselves be regulated by  $Ca^{2+}$  ions [205,206].  $NAD^+/NADH$  are not transported freely across mitochondrial membranes, and most biochemistry textbooks emphasize the importance of shuttle systems in the transport of reducing equivalents into mitochondria. Nevertheless, there is an intramitochondrial pool of  $NAD^+/NADH$  which may or may not be distinct in size from the cytosolic pool, and a limited transport of this co-factor must be possible. Finally, amino acids, acetate, glycerol-phosphate, keto acids, and many other metabolites must be able to enter or exit mitochondria. These latter transport systems and shuttles are more appropriately considered in Chapter 6 on metabolism.

It is generally assumed that pores in the outer membrane formed by an abundant mitochondrial protein named porin make the outer membrane highly permeable to most small molecules (<4000–5000 kDa). Thus, the inner mitochondrial membrane is the major permeability barrier for small molecules between the cytosol and the mitochondrial matrix.

The flux of protons through the inner membrane has been discussed extensively in the previous sections. Protons are pumped out of the matrix by the operation of the electron transport chain, and they return via complex V, and through various “leakage” channels, uncouplers, symporters and antiporters. In *in vitro* systems protons can be exchanged for potassium ions by the use of the ionophore nigericin. The elucidation of the precise path of a proton through the various complexes remains a challenging problem. An interesting mitochondrial proton pump which has received less attention (at least in textbooks) is the nicotinamide nucleotide transhydrogenase. The bovine enzyme is an integral membrane protein made up of two identical monomers of 109,065 kDa. An N-terminal domain (430 residues) and a C-terminal domain (200 residues) flank a hydrophobic central domain which is predicted to have 14 trans-membrane helices. The hydrophilic end-domains extend into the matrix, where the N-terminal domain binds NAD(H), and the C-terminal domain binds NADP(H). In the

process of transhydrogenation involving a hydride transfer from one co-factor to the other protons are pumped across the inner membrane.



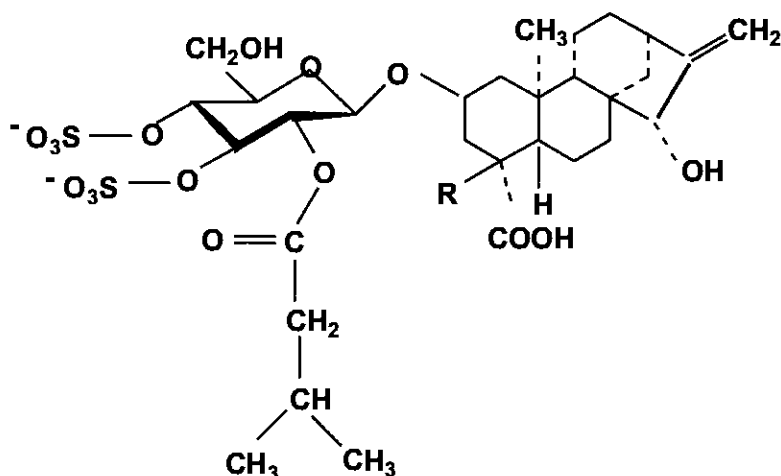
As discussed in more detail in a review by Hatefi and Yamaguchi [207], this transhydrogenase is a unique proton pump and a potentially powerful model system to understand how a simple protein can translocate protons across a membrane driven by conformational transitions which in turn are induced by a chemical reaction on one side of the membrane, and by the different binding energies for substrates and products.

#### 5.6.4.1 *The ADP/ATP Translocator*

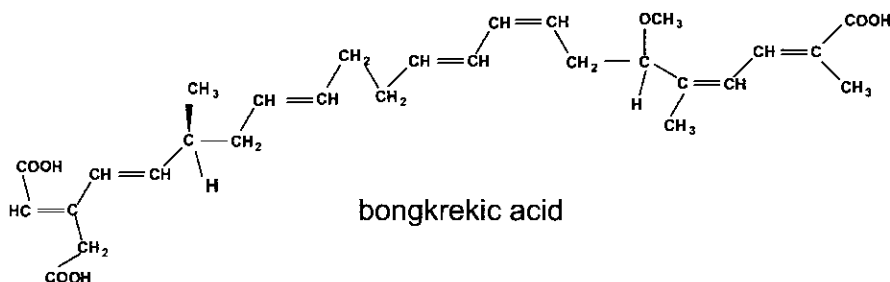
The ADP/ATP translocator (adenine nucleotide translocase [ANT]) is an interesting integral membrane protein with a rather unique function in mitochondria. Its properties, shared with the uncoupling protein, UCP-1, and with what is believed to be a family of mitochondrial carriers, have already been emphasized [196]. To repeat: The protein is an integral membrane protein with a molecular mass of approximately 32 kDA. There are three repeats of about 100 amino acids, each of which contains two shorter motifs representing transmembrane helices. The protein therefore is localized in the membrane with six transmembrane helices, and both the N-terminal and the C-terminal are on the same side, facing the cytosol. The loops on the matrix side contain an exceptionally polar combination of about 40 side chains. It is likely that a dimeric structure makes up the functional carrier with a twofold axis of rotational symmetry which has also been proposed to form the channel through which ADP/ATP translocation occurs.

A distinguishing feature of the translocator/carrier and UCP family is the ability to bind purine nucleotides. In the UCP-1 the nucleotide is a regulatory ligand controlling unidirectional transport of other small ions ( $\text{H}^+/\text{OH}^-$ , or  $\text{Cl}^-$ ), while in the ADP/ATP translocator the purine nucleotide is in fact the substrate and activator of translocation. UCP-1 binds the regulatory ligand only on the outside (cytoplasmic side) of the inner membrane. The translocator must bind nucleotides on either side, and the net reaction (ADP imported, or ATP exported) is driven by the membrane potential,  $\Delta\Psi$ , since ATP has one more negative charge than ADP.

Two highly specific inhibitors have been employed in the study and in the purification of the ADP/ATP translocator. These inhibitors do not bear much resemblance to each other or to ATP, except for the presence of three or four negative charges. Binding sites for the inhibitor and ATP are similar or overlapping, since binding of one precludes the binding of the other. Carboxyatractylate is derived from plants, and bongkreikic acid has been isolated from certain *Pseudomonas* strains. Their structures are shown in Figure 5.30. They differ in their ability to penetrate the inner membrane. Atractylate is restricted to the outside and hence binds to the cytoplasmic side of the carrier, whereas the bongkreikic acid can enter the mitochondria, and it binds only to the matrix side. The use of radiolabeled or fluorescent inhibitors can help in estimating the number of carrier sites per mitochondrion (it is generally a very abundant pro-



atractyloside  $R = H$   
 carboxyatractyloside  $R = COOH$



**Figure 5.30** A: Atractyloside. B: Bongkreikic acid.

tein—up to 15% of total protein). With the atractylate bound, the carrier can be stabilized during purifications and protected against enzymatic degradation [see Reference 196 for review and earlier references].

Most interesting and revealing, however, has been the observation that no ternary complexes have been found, that is, the inhibitors prevent not only the binding of ATP, or ADP, but both inhibitors cannot bind at the same time, even though they bind to opposite sides of the carrier. The interpretation by Klingenberg was that the carrier is alternately open for the substrate on one side or the other. It is postulated to exist in two conformational states, and during the transition from one state to the other the substrate is translocated across the membrane. In fact, it was further hypothesized that the transition between the two states (c-state and m-state) is possible only with the substrate (either ATP or ADP) present (“induced transition fit theory of carrier transport



catalysis"). According to this model the inhibitors can bind to the open faces of two opposing "ground states" of the carrier, stabilizing a conformation which is incapable of binding the adenine nucleotide on the other side.

#### 5.6.4.2 *Cation Transport*

The most significant cations other than protons are  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , and the trace metals  $\text{Fe}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ , and others. Because of its special importance in specific regulatory mechanisms, the uptake and release of  $\text{Ca}^{2+}$  will be considered under its own heading below. One primary mechanism of cation uptake/exchange involves antiporters, and several such proteins with distinct specificities have been described [208,209].  $\text{Na}^+$  ions can traverse the inner membrane via an apparently unregulated  $\text{Na}^+/\text{H}^+$  antiport, or a slightly more complex  $\text{Na}^+/\text{Ca}^{2+}$  antiport (see below). The  $\text{Na}^+$  ion concentration is generally low in the cytosol, and it is also kept low in the mitochondrial matrix. The presence of the  $\text{Na}^+/\text{H}^+$  antiport keeps the electrochemical  $\text{Na}^+$ -gradient equal to the  $\text{H}^+$ -gradient.  $\text{K}^+$  ions have a major role in maintaining mitochondrial volume, and their uptake is achieved by three mechanisms: an electroneutral  $\text{K}^+/\text{H}^+$  antiporter that may be regulated by free  $\text{Mg}^{2+}$  in the matrix, a  $\text{K}^+$  channel activated by ATP, and a leakage driven by the high electrochemical gradient across the inner membrane. The purified  $\text{mitK}_{\text{ATP}}$  channel has been studied in synthetic lipid bilayers to prove that regulatory nucleotides (and also acyl CoA esters) bind to the cytosolic side of the membrane [210,211]. Thus, a balance must be maintained between an outward flux via the antiporter driven by the proton gradient, and an inward flux through the leak and channel driven by the electrochemical gradient. The precise physiological significance of the ATP-sensitive channel remains to be fully explained, but it has been suggested that it serves in regulated energy dissipation at times of low ATP demand by the cell, when the flow and metabolism of reducing equivalents between matrix and cytosol has to be maintained. Mitochondrial volume has been shown to influence the rate of electron transport and respiration [208,212].

Uniports for  $\text{Na}^+$  and  $\text{K}^+$  have also been identified, but their characteristics and functional importance are still under investigation. Similarly, the passage of  $\text{Mg}^{2+}$  ions through the inner mitochondrial membrane is still poorly understood [213]. It appears that mitochondria take up  $\text{Mg}^{2+}$  through membrane leakage channels driven by the membrane potential, rather than a specific transporter [214]. Interesting speculations have centered on the possibility of hormonal control of  $[\text{Mg}^{2+}]$  in both the cytosol and matrix.

#### 5.6.4.3 *Transport of Calcium and Its Physiological Role*

Intramitochondrial free calcium  $[\text{Ca}^{2+}]_{\text{m}}$ , and the mechanisms associated with its control have been examined extensively, since its recognition as a potential mediator of a variety of metabolic activities. Additional importance is derived from the recognition that certain pathological conditions may be associated with abnormal  $\text{Ca}^{2+}$  levels in mitochondria [206]. The nature of the transporters, the bioenergetics of transport, and the regulation of  $\text{Ca}^{2+}$  transport all have been investigated since the discovery of mitochondrial Ca transport in the early 1960s [215]. The existing membrane potential favors influx, and efflux is energetically uphill. An expert discussion of the energet-

ics of  $\text{Ca}^{2+}$  transport is presented in a review by Gunter and Gunter [205]. From typical values for  $\Delta\Psi$  and internal and external  $[\text{Ca}^{2+}]$  it is estimated that  $\sim 29\text{kJ/mol}$   $\text{Ca}^{2+}$  are required to get  $\text{Ca}^{2+}$  out of mitochondria. From another point of view this considerable free energy difference explains how mitochondria can become intracellular “sinks” or reservoirs for  $\text{Ca}^{2+}$ , leading to the challenge to understand how they contribute to the control to the overall cytosolic level of this ion.

A major player in  $\text{Ca}^{2+}$  transport in vertebrate mitochondria is a uniporter that can also transport  $\text{Mn}^{2+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Pb}^{2+}$  in decreasing order of selectivity, although, curiously,  $\text{Mg}^{2+}$  uptake may not involve this route. The conclusion that it is a uniporter rests on a variety of experiments summarized in Reference 205. The membrane potential (negative inside) is essential, favoring the net charge transfer of 2, that is,  $\text{Ca}^{2+}$  diffuses down the  $\text{Ca}^{2+}$  electrochemical gradient, and in the process diminishes the membrane potential. Detailed kinetic studies have been interpreted to show the existence of two sites for  $\text{Ca}^{2+}$  on the uniporter: one for activation and the second for the actual transport. Another interesting observation is that this uniporter does not appear to allow  $\text{Ca}^{2+}$  ions to pass down a chemical gradient alone, that is, a membrane potential is required to induce a conformation necessary for transport.

Two distinct  $\text{Ca}^{2+}$  efflux mechanisms were recognized in vertebrate mitochondria. It was a challenge to prove that the  $\text{Na}^{+}$ -independent mechanism was not an artifact or simply a reversed uniport [reviewed in Reference 205]. It now appears established that  $\text{Ca}^{2+}$  efflux is accompanied by proton influx, and a ratio  $1\text{Ca}^{2+}/2\text{H}^{+}$  appears to be favored. However, bioenergetic considerations conclude that it cannot be a simple passive exchanger, and some additional input of energy from the electron transport chain must be postulated. The energy coupling mechanism is still obscure. A  $\text{Na}^{+}$ -dependent mechanism can be distinguished on the basis of certain ion selectivities ( $\text{Sr}^{2+}$  but not  $\text{Mn}^{2+}$ ), stimulation by  $\text{Na}^{+}$ , spermidine and spermine, and inhibition by a variety of inhibitors. There is still uncertainty about whether it is a passive, nonelectrogenic  $\text{Ca}^{2+}/2\text{Na}^{+}$  exchanger, an active mechanism, or even a  $\text{Ca}^{2+}/3\text{Na}^{+}$  exchanger, with the evidence tilting in favor of a  $\Delta\Psi$ -dependent electrophoretic antiport [216].

Our understanding of the physiological role of mitochondrial  $\text{Ca}^{2+}$  transport is still expanding [206]. It has long been recognized that mitochondria can constitute a buffering compartment with a role in modulating cytosolic calcium,  $[\text{Ca}^{+}]_{\text{cytosol}}$ . A relevant question addresses whether transport in and out of mitochondria is sufficiently rapid to keep up with rapid, pulsed changes in cytosolic  $[\text{Ca}^{2+}]$ , found, for example, in beating cardiomyocytes or in nerve cells engaged in active signaling (exocytosis triggered by elevated  $[\text{Ca}^{+}]_{\text{cytosol}}$ ). A second major insight is that two prominent mitochondrial dehydrogenases (pyruvate dehydrogenase and  $\alpha$ -ketoglutarate dehydrogenase) are activated by  $[\text{Ca}^{2+}]_{\text{m}}$ . It should be noted that the critical concentrations are those of the free  $\text{Ca}^{2+}$ . Estimates are that only one part of  $10^3$  of total mitochondrial  $\text{Ca}^{2+}$  is free, and this fact must also be considered in the context of the buffering capacity of mitochondria. The stimulation of the dehydrogenases increases the  $\text{NADH}/\text{NAD}^{+}$  ratio and hence oxidative phosphorylation. One can consider a plausible scenario in which increased  $[\text{Ca}^{+}]_{\text{cytosol}}$  in stimulated skeletal or cardiac muscle is rapidly coupled to increased  $[\text{Ca}^{2+}]_{\text{m}}$  and hence increased rates of oxidative phosphorylation. Such a mechanism could serve to signal increased energy demand in the cy-

tosol to the OXPHOS machinery. A more detailed discussion of the experimental evidence in support of this view is presented by Hansford [206]. The use of fluorescent  $\text{Ca}^+$  indicators (e.g., fura-2) developed by Tsien and Poenie [217] has revolutionized the measurement of  $[\text{Ca}^+]_{\text{cytosol}}$ , and has even been applied to isolated mitochondria. More recently, recombinant aequorin has been targeted to mitochondria to serve as a sensitive indicator of  $[\text{Ca}^{2+}]_{\text{m}}$  [218]. The combination of fura-2 and mitochondrial aequorin in single cells permitted simultaneous measurements of  $[\text{Ca}^+]_{\text{cytosol}}$  and  $[\text{Ca}^{2+}]_{\text{m}}$ , with intriguing results [reviewed in Reference 219]. Raising  $[\text{Ca}^+]_{\text{cytosol}}$  by influx from extracellular  $\text{Ca}^{2+}$  affected  $[\text{Ca}^{2+}]_{\text{m}}$  less and at a slower rate than an increase in  $[\text{Ca}^+]_{\text{cytosol}}$  by a release ( $\text{IP}_3$ -induced) from intracellular stores (ER). Microdomains inside of cells near  $\text{Ca}^{2+}$  release sites may explain the observed behavior.

#### 5.6.4.4 *The Mitochondrial Permeability Transition*

The mitochondrial permeability transition (MPT) was originally defined as sudden increase in the inner membrane permeability to solutes of molecular mass less than ~1500 Da. It is now believed to be due to the opening of a mega channel, which is referred to in some publications as the mitochondrial permeability transition pore (MTP). It has received particular attention in recent years because pore opening is sensitive to cyclosporin A (see below), and because it has been implicated in the pathway of apoptosis (see Chapter 7.4). The transition is detectable and measured with isolated mitochondria in protein-free buffers by an uptake of radioactive molecules such as sucrose, by a change in optical density due to swelling, and even by patch clamp techniques [144,220]. The original observations were made with animal mitochondria (beef heart, rat liver), but recent publications suggest that yeast mitochondria are also capable of forming a transition pore. However, the regulation of this pore in yeast may be different [221].

Several broad questions can be raised, and answers to these questions are beginning to accumulate. The discussion will focus on the following issues: 1) What components of the inner membrane constitute the pore? 2) What mechanisms regulate the opening and closing of the pore? 3) What is the physiological significance of the operation of this mega channel? To some extent the answers are interrelated.

The exact composition of the pore is still not completely clear, except for a general agreement that the adenine nucleotide translocase (ANT) is a participant in channel formation, perhaps by forming complexes with outer membrane proteins to create contact sites and hence to stabilize and/or enlarge the channel. The peripheral benzodiazepine receptor and porin in the outer membrane have been implicated, and there is speculation about the inclusion of Bcl2, cardiolipin synthase, 3- $\beta$ -hydroxysteroid isomerase, and phospholipid hydroperoxide glutathione peroxidase [222]. On the cytosolic side hexokinase may also be associated, and on the matrix side the participation of cyclophilin D is suggested by the sensitivity to cyclosporin.

The channel can be opened when  $\text{Ca}^{2+}$  is accumulated in the matrix by exposure of mitochondria to high concentrations of  $\text{Ca}^{2+}$  (generally above physiological levels), and the sensitivity to  $\text{Ca}^{2+}$  is heightened under various conditions including oxidative stress, adenine nucleotide depletion, elevated phosphate concentrations, and low membrane potential,  $\Delta\Psi$ . The role of nucleotides points to an involvement of ANT, but

a more direct indication is the effect of inhibitors of ANT on the MPT. Inhibitors which stabilize the “c” conformation of ANT (e.g., atractyloside) favor the MPT, while inhibitors stabilizing the “m” conformation (e.g., bongkrekic acid) also inhibit pore opening. A high  $\Delta\Psi$  or a low pH prevent the transition [223]. Of special interest was the observation that submicromolar concentrations of cyclosporin A are very effective in keeping the pore from opening. The target for cyclosporin is the matrix peptidyl-proline cis-trans isomerase, a member of the cyclophilin family. One interpretation was that the (soluble) matrix isomerase may interact with an integral membrane protein, and when triggered by  $\text{Ca}^{2+}$ , the pore opening is induced. Cyclosporin A would interfere with this required interaction. A further speculation was that the relevant IMP was the ANT [224]. The idea was supported by the observed attachment of cyclophilin to the inner membrane under certain conditions (oxidative stress, thiol reagents, increased matrix volume) which favor or enhance pore opening [225;226].

The combined observations have served to support a variety of potential functions for the MTP that may not be exclusive [222]:

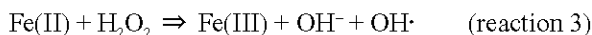
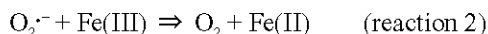
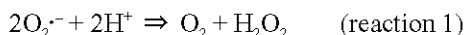
1. A voltage sensor
2. A matrix pH sensor
3. A divalent cation sensor (role in calcium homeostasis [e.g., 227])
4. A sensor of adenine nucleotide concentrations
5. A sensor of the redox state of the pyridine nucleotide pool
6. A thiol sensor (redox status of glutathione).

It is clear that a prolonged opening of a number of pores, or the simultaneous opening of many pores would deenergize and inactivate mitochondria rapidly, and it may be that such an event is associated with the irreversible breakdown of  $\Delta\Psi$  observed in the course of apoptosis (see Chapter 7.4). However, it is at this time still a matter of much speculation whether the MTP makes a significant contribution to mitochondrial activities under physiological conditions. It could be imagined as a safety valve under stressful conditions, but the lack of specificity for any small molecular weight solute precludes any fine-tuning for specific ions or solutes.

## 5.7 REACTIVE OXYGEN SPECIES

The major reactive oxygen species (ROS) are the superoxide radical,  $\text{O}_2^-$ , hydrogen peroxide,  $\text{H}_2\text{O}_2$ , and the hydroxyl radical,  $\text{OH}\cdot$ . When protonated, the superoxide radical becomes a hydroperoxyl radical ( $\text{pK}_a = 4.8$ ). In fact, the only very reactive species among them is the hydroxyl radical which is believed to be responsible for most of the actual damage done to biological macromolecules. It is so reactive that damage is restricted to a small radius limited by diffusion of the radical. The risk posed by the other two species comes primarily from reactions which ultimately lead to the formation of the hydroxyl radical. Attempts to react the superoxide in aqueous solution

directly with biologically relevant molecules have been frustrated. Therefore, the proposal has been made that a series of reactions ultimately result in hydroxyl radical formation [228,229]:



summing reactions 2 and 3 yields:



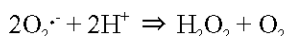
Reactions 2 and 3 have been postulated as intermediate reactions because reaction 4 has been shown to depend on the presence of redox metals. Reaction 3 is the Fenton reaction, and it emphasizes again that hydrogen peroxide is by itself less reactive, but upon encountering the ubiquitous metal ions in a cell it generates the highly reactive hydroxyl radical. Instead of Fe(II), reaction 3 can also take place with Cu(I) as the reducing agent.

Complex IV of the mitochondrial electron transport chain is not the only site in a cell where oxygen can be activated to the superoxide radical [229]. In fact, it might be argued that electrons “leaking” from the electron transport chain to oxygen at high potential upstream sites are responsible for the majority of ROS produced in mitochondria. The superoxide radical is also generated “accidentally” by reactions of oxygen at microsomal cytochromes P450, and by less well defined or understood reactions (autooxidation reactions) involving catecholamines, ascorbic acid, and reduced flavins, often with the participation of a metal ion. The toxicity of such compounds has been exploited in some physiologically useful reactions. The best known example is the generation of superoxide radicals, hydrogen peroxide and HOCl by activated macrophages, presumably to kill their target cells (bacteria, fungi, etc.) before phagocytosis. Several peroxisomal oxidases generate hydrogen peroxide, for example, D-amino acid oxidase and xanthine oxidase. In some systems the superoxide radical has been proposed as an intercellular signal. In a more indirect way, the  $\text{O}_2^{\cdot-}$  ion can remove nitric oxide, another free radical and an intercellular messenger in neurotransmission, in the immune response, and in the regulation of smooth muscle contraction. Finally, reactive oxygen species have been assigned a central role in the mechanisms of apoptosis (see Chapter 7.4), with arguments still unresolved about whether they are responsible for cell killing, or whether their increased generation is the consequence of another damaging event affecting mitochondria and perhaps other cellular constituents.

A variety of protective enzymes have evolved to guard against damage by ROS, or even to repair damage (e.g., in DNA) resulting from their reactivity. Present day aerobic organisms presumably arose from anaerobes by evolving antioxidant defense mechanisms among others, as the oxygen content of the atmosphere on earth increased to the present 21%. The existence of antioxidant defense mechanisms in aer-

obic organisms is therefore crucial for survival or longevity. Activity in the field concerned with ROS and antioxidants continues to be high, because many fundamental questions remain unanswered.

One of the main mechanisms in cells is to sequester iron and copper ions in such a way that they cannot react readily with superoxide or hydrogen peroxide. Availability and location of "free" metal ions in a cell thus can determine the site of damage, because the hydroxyl radical formed is too short-lived to diffuse far from its site of origin. For example, metal ions bound to DNA can initiate free radical damage in the nucleus. The potential significance of the superoxide radical was underlined by the discovery of superoxide dismutase (SOD) by McCord and Fridovich [230]. These enzymes greatly accelerate the dismutation reaction:



Catalases (see below) then convert the hydrogen peroxide to water and oxygen. Two SODs have since been characterized in some detail. A cytosolic form is distinguished by having copper and zinc at the active site (Cu,Zn-SOD), while a mitochondrial form contains manganese (Mn-SOD). The cytosolic SOD is encoded by a gene on human chromosome 21, and tissue levels of Cu,Zn-SOD are elevated ~50% in Down syndrome patients. Since transgenic mice overexpressing human SOD also have some neuromuscular abnormalities reminiscent of Down syndrome, it has been concluded that too much of this protective enzyme can also be deleterious.

Catalases are enzymes characterizing peroxisomes, where they remove hydrogen peroxide generated by peroxisomal oxidations. However, the bulk of the  $\text{H}_2\text{O}_2$  in mammalian cells is removed by glutathione peroxidases. A selenocysteine residue is required at their active site, where  $\text{H}_2\text{O}_2$  is used to oxidize glutathione (GSH) to oxidized glutathione (GSSG). GSH can be regenerated from GSSG by the oxidation of NADPH, catalyzed by glutathione reductase.

It is generally considered that the generation of ROS and the activity of antioxidant defenses may have to be carefully balanced *in vivo*. A very slight imbalance in favor of ROS may be responsible for normal aging. A more severe imbalance is said to result in oxidative stress. Oxidative stress may result in the induction of antioxidant defense enzymes and mechanisms, but beyond a certain level it can increase intracellular calcium levels and free iron levels, accentuating the problem to catastrophic proportions. A major unsolved and still controversial issue is whether increased ROS production is a primary consequence of a mitochondrial mutation, or whether a primary defect outside of the mitochondria is responsible for an abnormal respiratory chain and incomplete reduction of oxygen.

## 5.8 ROLE OF SPECIFIC LIPIDS

Over the years the purification of various complexes from the electron transport chain or complex V, and also of individual carriers such as the nucleotide transporter, has yielded preparations which not unexpectedly contained various lipids, since efforts

were made to retain biological activity and native conformations. Consistently such preparations contained cardiolipin [e.g., 231–234]. Cardiolipin is found exclusively in mitochondria (see Section 6.6), and it became “conventional wisdom” to postulate that cardiolipin may be essential for either the assembly of these complexes in the membrane or their maintenance in their functional conformation [e.g., 235,236].

An experimental test for this idea became possible when genetic approaches were used to eliminate cardiolipin by mutating the specific enzymes required for its biosynthesis (see Section 6.6). In mammalian cells in tissue culture this has been achieved by Ohtsuka and colleagues [237,238], who isolated Chinese hamster mutants with a defect in phosphatidylglycerophosphate synthase. Such cells had depleted cardiolipin levels and exhibited mitochondrial dysfunction. Complex I appeared to be the most seriously affected. By contrast, a recent study [239,240] made with a yeast mutant containing a defective cardiolipin synthase gene, *cls1*, and hence no cardiolipin in its membranes, gave the surprising result that such mutants were viable and capable of respiration, that is, they grew in the presence of either non-fermentable or fermentable carbon sources. What, if any, compensatory changes in lipid composition have occurred remains to be seen.

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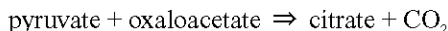
## **METABOLIC PATHWAYS INSIDE MITOCHONDRIA**

### **6.1 INTRODUCTION**

Until about the middle of this century investigators preparing crude extracts from muscle and liver homogenates for the purpose of biochemical and enzymatic studies did not pay any attention to subcellular compartmentalization and its potential significance. Thus, when A. Claude and C. de Duve at the Rockefeller Institute perfected techniques for isolating relatively pure mitochondria, a new era in biochemistry began. In a short time span the enzymes for several important metabolic pathways were localized within the mitochondria, starting with the pioneering work of A.L. Lehninger and E.P. Kennedy on fatty acid oxidation. The citric acid cycle (TCA cycle) had already been postulated by H. Krebs in 1937, building on earlier work by A. Szent-Györgyi and others, but again, Lehninger and Kennedy, taking advantage of having pure, isolated mitochondria, demonstrated in 1948 that the entire citric acid cycle takes place inside the mitochondria. Together with the spectroscopic identification of cytochromes in mitochondria these discoveries led to the recognition of mitochondria as the “powerhouse of the cell.” Mitochondria became the first subcellular organelles outside of the nucleus to be functionally characterized. It took not much longer to identify lysosomes as the organelle containing hydrolytic enzymes, although the role of lysosomes in the overall economy of cells did not become apparent until later. Peroxisomes were also characterized by the discovery of a few key enzymes such as catalase, but the description of their full complement of enzymes took several decades. Finally, the ER and Golgi apparatus were placed firmly in the secretory pathway by the classical pulse-chase experiments of J.D. Jamieson and G. Palade, and the pioneering biochemical studies of D. Sabatini and G. Blobel. The detailed biochemical and functional description of the ER and Golgi constitute highlights of the past two decades.

## 6.2 THE KREBS CYCLE

A description of the citric acid cycle is found in all elementary and advanced textbooks, and its formulation ranks among the major achievements in the study of metabolism. It was the second cycle to be postulated by Krebs, since he had also discovered the urea cycle a few years earlier. When it was first proposed by Krebs in 1937, there were still some major areas of uncertainty, especially in the production of citrate, which Krebs formulated as the following reaction:



It was not until coenzyme A had been discovered by N.O. Kaplan and F. Lipmann in 1945 that S. Ochoa and F. Lynen were able to show in 1951 that acetyl-CoA was the intermediate in the reaction with oxaloacetate to form citrate.

Today the reactions of the citric acid cycle are firmly established, and the cycle itself occupies a central position on any metabolic chart. When it is first presented to students, it is frequently emphasized that the constituents of the cycle act catalytically, that is, are themselves not being increased or consumed. Subsequently it becomes necessary to add to the catabolic functions of the cycle anabolic functions as well, that is, cycle intermediates are being drained off to form amino acids and numerous precursors for other biosynthetic reactions. For example, succinyl-CoA is a precursor for heme synthesis. At the same time, the  $\alpha$ -keto-dicarboxylic acids of the cycle can be formed from the transamination of amino acids, and oxaloacetate can also be produced from the carboxylation of phosphoenolpyruvate in certain tissues such as liver.

Reactions of the TCA cycle are also essential for complementing the reactions of the glyoxylate cycle in plants. This cycle has been speculated to be an evolutionary precursor for the TCA cycle, still sharing with it some reactions. However, the significant aspect of the glyoxylate cycle is that a novel reaction can take place with isocitrate as the substrate. Instead of being followed by two decarboxylation reactions, isocitrate is split by isocitrate lyase to form succinate and glyoxylate. Glyoxylate condenses with a second acetyl-CoA to enter the cycle to form malate. The net result is the operation of a cycle which converts two acetyl groups to succinate. The glyoxylate cycle takes place in glyoxysomes, a distinct organelle of plants, related to peroxisomes, and found prominently in germinating seedlings. It permits the utilization of fats and oils stored in the seeds to make carbohydrates before plant growth and development has formed green leaves for photosynthesis. The succinate produced has to leave the glyoxisomes and enter mitochondria, where it is converted to oxaloacetate which can be withdrawn to form phosphoenolpyruvate for gluconeogenesis.

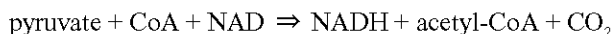
Is there anything left to discover about the citric acid cycle? All the enzymes have been purified from various organisms, and many of the corresponding genes have been or are being cloned. Catalytic mechanisms have been defined in considerable detail, and crystal structures of many enzymes are available. All but one enzyme of the citric acid cycle are soluble enzymes in the mitochondrial matrix. The exception is succinate dehydrogenase which is part of complex II and forms the peripheral membrane component of this complex (see Chapter 5). Its characteristics as an elec-



tron transfer complex have been discussed elsewhere in this volume. Structure–function analyses of this complex remain a major challenge which will not be completely resolved until the crystal structure is available. Understanding the assembly of the complex from precursors made in the cytosol and imported into mitochondria also remains a challenge.

For the theoretically inclined, the regulation of the fluxes of metabolites through the cycle presents a second challenge, especially in light of new insights about the “robustness” of metabolic pathways, and the emphasis on control coefficients for the various enzymes as opposed to the simpler view of rate limiting enzymes. How is the activity of the cycle adjusted to various conditions in a given tissue such as muscle, and how is the cycle’s capacity regulated in different tissues?

Before some answers to these questions can be proposed, a presentation of the cycle and a limited discussion of the individual steps is in order. It is logical to start with one reaction outside of the cycle which produces the important intermediate acetyl-CoA. The overall reaction is

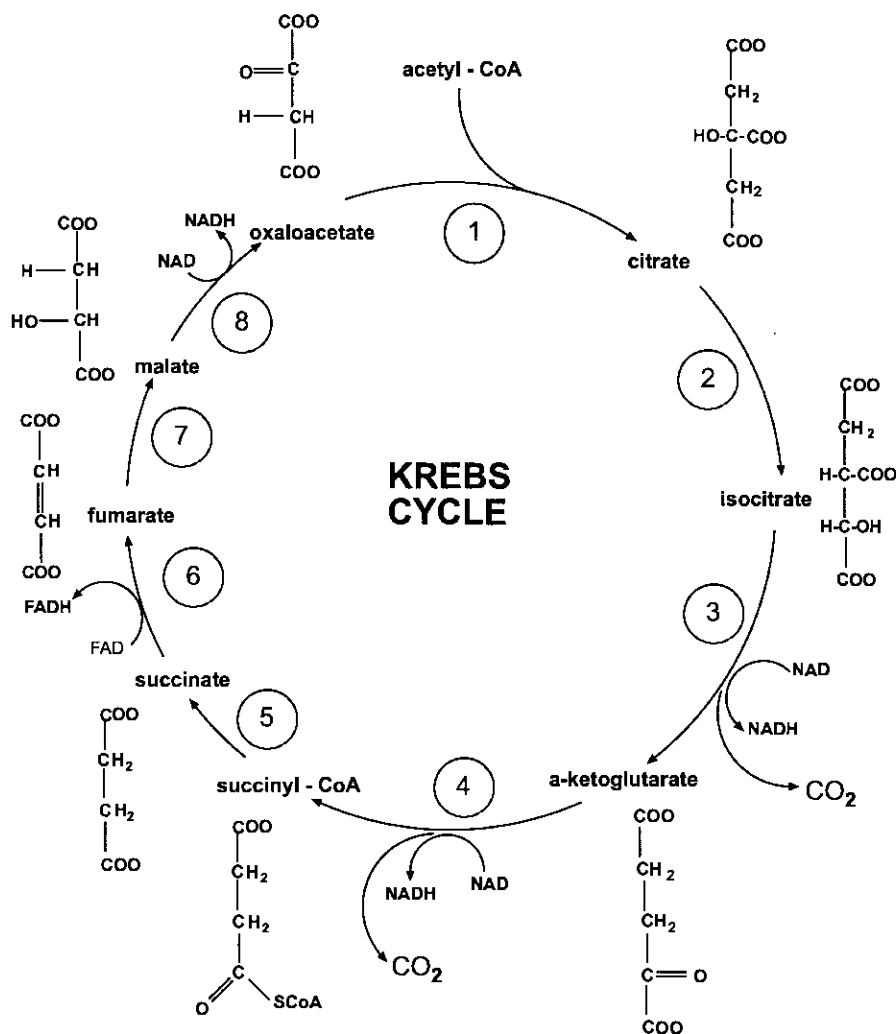


The enzyme involved is a multienzyme complex named pyruvate dehydrogenase, consisting of 132 subunits (30 E<sub>1</sub> dimers, 60 E<sub>2</sub> dimers, and 6 E<sub>3</sub> dimers in mammals) with a total molecular mass of several hundred thousand daltons. Five coenzymes are necessary: thiamine pyrophosphate, lipoamide, CoA, FAD, and NAD. Substrates and intermediates are shuttled between the subunits, facilitated by the highly organized, three-dimensional structure of this complex. For a detailed discussion of the structure and mechanism the reader is referred to standard biochemistry textbooks, or to the original literature.

Figure 6.1 presents an overview of the intermediates and their sequence in the citric acid cycle. The individual reactions have been numbered, and the following list presents the salient features of each reaction and the corresponding enzyme.

1. Citrate synthase catalyzes the condensation of acetyl-CoA with oxaloacetate to form citrate, a tricarboxylic acid from which a common name of the cycle has been derived. The x-ray structure of the enzyme has been determined, and considerable detail about the mechanism is known. The reaction can be described as a mixed aldol-Claisen condensation involving the stabilization of the enol form of acetyl-CoA as an intermediate. Our current understanding of enzymes and their active sites also makes it clear why a specific chiral carbon is formed with the carboxymethyl group from acetyl-CoA constituting the pro-S arm. A consequence of this stereochemistry is that the two carbons entering the cycle from acetyl-CoA will not be liberated as CO<sub>2</sub> during the first turn of the cycle.

2. Aconitase catalyzes the reversible interconversion between two structural isomers, citrate and isocitrate. Since citrate is prochiral, the two carboxymethyl groups of the symmetrical citrate are distinguishable, and the hydroxyl group is moved to the methylene carbon from oxaloacetate rather than to the methylene group of the original acetyl-CoA. The enzyme is of special interest because a non-heme iron sulfur cluster ([4Fe–4S]) participates in the active site of the enzyme, although no electron



**Figure 6.1** The tricarboxylic acid cycle (TCA cycle), or Krebs cycle. The enzymes catalyzing the eight steps are: 1, citrate synthase; 2, aconitase; 3, isocitrate dehydrogenase; 4,  $\alpha$ -ketoglutarate dehydrogenase; 5, succinyl-CoA synthase; 6, succinate dehydrogenase (SDH); 7, fumarase; 8, malate dehydrogenase. NAD is reduced by steps 3, 4, and 8; FAD is reduced by step 6. Two molecules of  $\text{CO}_2$  are produced in steps 3 and 4.

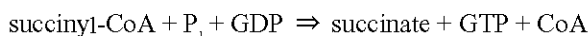
transfer takes place. A specific Fe(II) in the cluster serves to bind and stabilize the substrates and intermediates. More recently it was discovered that a cytosolic aconitase also serves as a sensor of iron in the cytosol. Before its identity was established, this iron-responsive protein (IRP) had been shown to interact with iron-responsive elements (IREs) on certain mRNAs. These IREs can be recognized as specific hairpin structures. When bound to IREs in the 5' untranslated region of the mRNAs for ferritin or 5-aminolevulinate synthase, it prevents translation of these mRNAs. Cytosolic

aconitase binds to IREs when the iron concentration is low; at high iron concentrations aconitase is more tightly folded around the [4Fe–4S] cluster and incapable of binding the mRNA. An IRE is also found in the 3' untranslated region of the transferrin receptor mRNA. Binding of aconitase (low iron) prevents turnover of the mRNA. At high iron concentrations degradation of the transferrin receptor mRNA is promoted. Thus, aconitase as the iron sensor controls the transport of iron into the cell, the storage of iron, a step in heme biosynthesis, and probably other pathways related to iron [see References 1–3 for recent reviews].

3. Isocitrate dehydrogenase occurs in two forms in mammalian tissues. An NAD-dependent form is found exclusively in mitochondria; a second form utilizes NADP and is localized both in mitochondria and the cytosol. The reaction produces the first of two CO<sub>2</sub> molecules produced per turn of the cycle, and it links a decarboxylation to the oxidation of an alcohol to a keto group. The first of three NADHs is also produced here.

4. The enzyme  $\alpha$ -ketoglutarate dehydrogenase is a multifunctional enzyme complex like the pyruvate dehydrogenase, and the third enzyme, dihydrolipoyl dehydrogenase is identical to that in PDH. The first two subunits are similar, with specificity for the succinyl- instead of acetyl- moiety, and the final product is another high-energy thioester, succinyl-CoA. NADH and CO<sub>2</sub> are the other products of the reaction. At this point two CO<sub>2</sub>s have been produced, accounting for the total input of two carbons from acetyl-CoA, but the stereochemical considerations related to the formation of citrate and the subsequent reactions can account for the observations that no radioactive CO<sub>2</sub> is produced from [<sup>14</sup>C]acetyl-CoA during the first turn of the cycle.

5. Succinyl-CoA synthetase carries out a substrate level phosphorylation with the slight twist that GDP is the phosphate acceptor:



There is persuasive evidence from isotope tracer experiments that the inorganic phosphate displaces the CoA to form succinyl-phosphate. The phosphate is transferred to GDP via a 3-phosphohistidine intermediate on the enzyme.

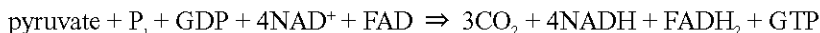
6. Succinate is oxidized to fumarate by the membrane-linked SDH complex. A large (70 kDa) flavoprotein forms the active site, and a covalently linked flavin becomes the hydrogen acceptor to form FADH<sub>2</sub>. The flavin is linked to a histidine side chain in a highly conserved portion of the protein. It is immediately reoxidized with the release of protons and the transfer of two electrons to the iron-sulfur centers of the iron protein subunit of SDH. The Ip subunit has three nonheme iron sulfur centers ([2Fe–2S], [3Fe–4S], and [4Fe–4S]) and constitutes a prime example of a protein conducting electrons in the absence of a heme group. The two integral membrane proteins of complex II are associated with a cytochrome, but it is still controversial whether this cytochrome participates in the conduit of the electrons to the ubiquinone (CoQ). Succinate dehydrogenase is the only Krebs cycle enzyme directly linked to the electron transfer chain. A more detailed discussion of this complex can be found in Chapter 5.

7. Fumarate is hydrated by the addition H<sub>2</sub>O to the double bond, catalyzed by fumarase. Controversy about the mechanism has centered on the question whether the

addition of  $\text{OH}^-$  is the first step, leading to the formation of a carbanion which appears now to have been confirmed by  $^{18}\text{O}$  exchange experiments.

8. The final reaction catalyzed by malate dehydrogenase constitutes the last oxidation step and hence production of another NADH.

A final accounting of all the reactions with the inclusion of the pyruvate dehydrogenase reactions leads to the following summation:



It should be reemphasized that pyruvate (C3) has become converted to three  $\text{CO}_2$  molecules without any consumption of oxygen. If a steady supply of NAD was available, glucose could thus be broken down to carbon dioxide in the absence of oxygen. The cost of  $\text{NAD}^+$  makes this impossible, and in reality it has to be obtained by recycling. Hence oxidation of NADH by complex I of the electron transfer chain is essential to keep the citric acid cycle going. Accumulation of NADH causes a strong inhibition of pyruvate dehydrogenase and  $\alpha$ -ketoglutarate dehydrogenase, and my laboratory has isolated mammalian cell mutants with defects in the electron transfer chain based on the almost total inhibition of the Krebs cycle [4]. Substrate and product concentrations are the immediate controlling factors determining the flux through the cycle, and ATP, ADP, and  $\text{Ca}^{2+}$  are allosteric effectors of citric acid cycle enzymes. Overall, oxygen consumption, NADH oxidation, and ATP synthesis are not only tightly coupled to each other, but also to the citric acid cycle by the above mechanisms.

In the end, Lavoisier was correct in viewing respiration as a combustion, but little did he suspect of the many steps necessary to make this “burning” of glucose in a living cell such a controlled process. The many steps involved insure not only control over a “slow burn,” but also permit the efficient capture of the free energy released in the process, and its interconversion and utilization in many biological processes. It all started with attempts to understand respiration, followed by a curiosity about the biochemistry and energetics of muscular contraction. As a final thought it may be worthwhile to be made aware that an average person turns over more than his/her own bodyweight of ATP per day, depending on the level of activity.

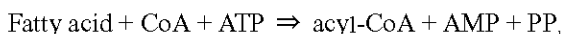
### 6.3 FATTY ACID METABOLISM

Fatty acid oxidation was one of the first metabolic pathways firmly localized in mitochondria by the pioneering studies of A.L. Lehninger and E.P. Kennedy in the late 1940s. Since fats and fatty acids derived from them are obvious energy sources, their degradation in mitochondria must have been suggestive. Soon thereafter the localization of the Krebs cycle enzymes in the same organelle allowed the integration of fatty acid oxidation with the TCA cycle via the important intermediate acetyl-CoA. Peroxisomes had not been recognized at that time, and therefore the discovery of  $\beta$ -oxidation of fatty acids in peroxisomes did not confuse the issue. It is now known that peroxisomes in animal cells “specialize” in the degradation of very long fatty acids (>22 carbon atoms), and they may even just shorten them to be accepted by the mi-

tochondrial system. In plant cells, however, fatty acid degradation is restricted to peroxisomes and glyoxysomes. The latter are specialized peroxisomes with special significance in metabolism during seed germination.

Before considering the details, it will be instructive to take a more global view. The fatty acids are first made available in the cytosol, for example from the hydrolysis of cholesterol esters of low density lipoprotein (LDL) in lysosomes, or from the degradation of phospholipids by lipases. Their transport into mitochondria is not a trivial issue (see below). The final product of their degradation is acetyl-CoA which can feed into the Krebs cycle. Under different conditions fatty acids can also be synthesized, also starting from acetyl-CoA. The biosynthesis of fatty acids does not occur simply by the reversal of the reactions of the  $\beta$ -oxidation, an observation that generally applies to other pathways such as glycolysis and gluconeogenesis. Different enzymes are utilized, and most significantly, the biosynthesis of fatty acids takes place in the cytosol. During elongation in the cytosol the acyl group is attached to a large multifunctional protein (in animal cells) taking the place of CoA.

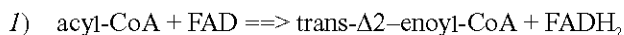
Fatty acid degradation not only generates acetyl-CoA as the final product, but intermediates are also attached to CoA during the entire process. Thus, the first step in fatty acid degradation requires an activation



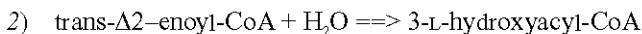
Several acyl-CoA-synthetases (thiokinases) serve in this activation which takes place in the cytosol. To reach the mitochondrial matrix, a shuttle system has evolved which is shown in Figure 6.2. The acyl group is transferred to carnitine, liberating CoA. Acyl-carnitine is transported into the mitochondria by a specific carrier protein, and once inside, the trans-esterification is reversed to form carnitine and acyl-CoA. The carrier protein is operating like an antiporter, transporting free carnitine out of the mitochondria.

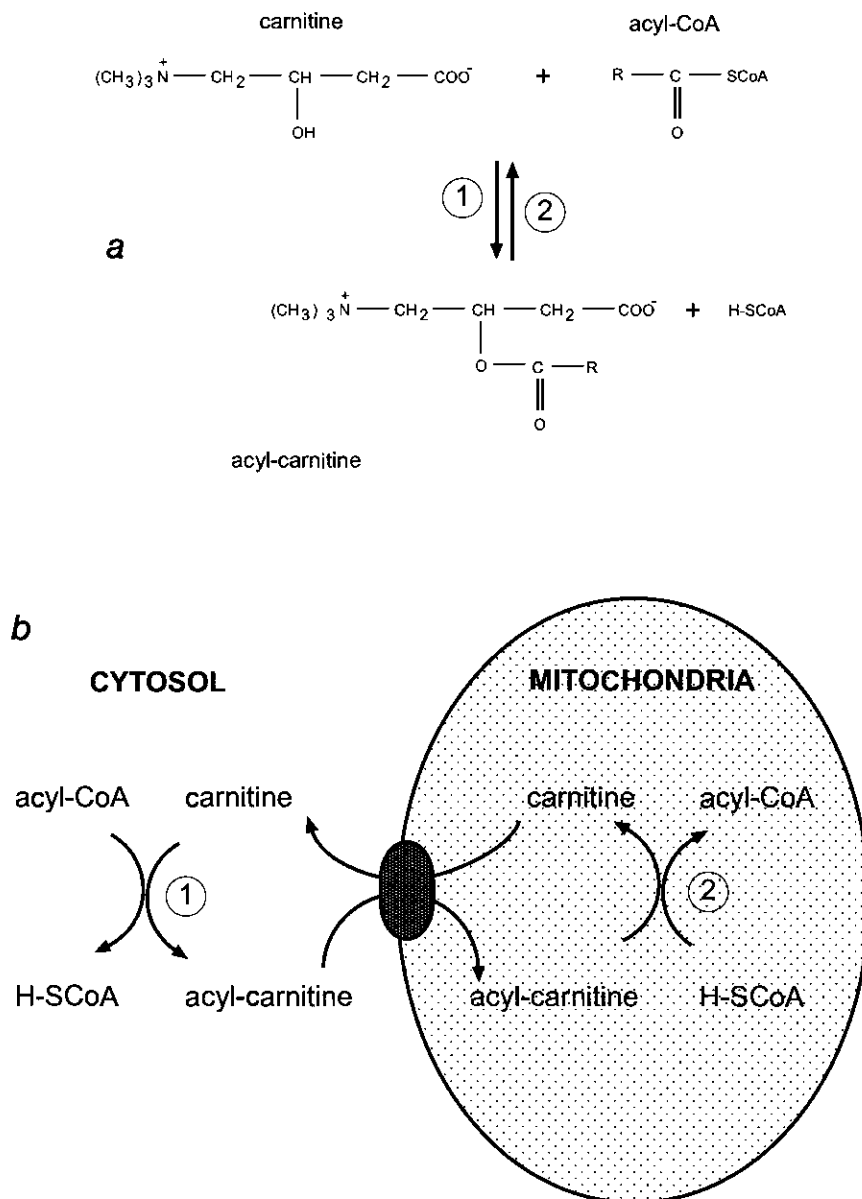
The  $\beta$ -oxidation reactions (Figure 6.3) are well known and found in all standard biochemistry textbooks. A brief listing of all reactions and some select comments follow.

The first reaction is an elimination of hydrogen to form a double bond between the  $\alpha$  and  $\beta$  carbons of acyl-CoA.

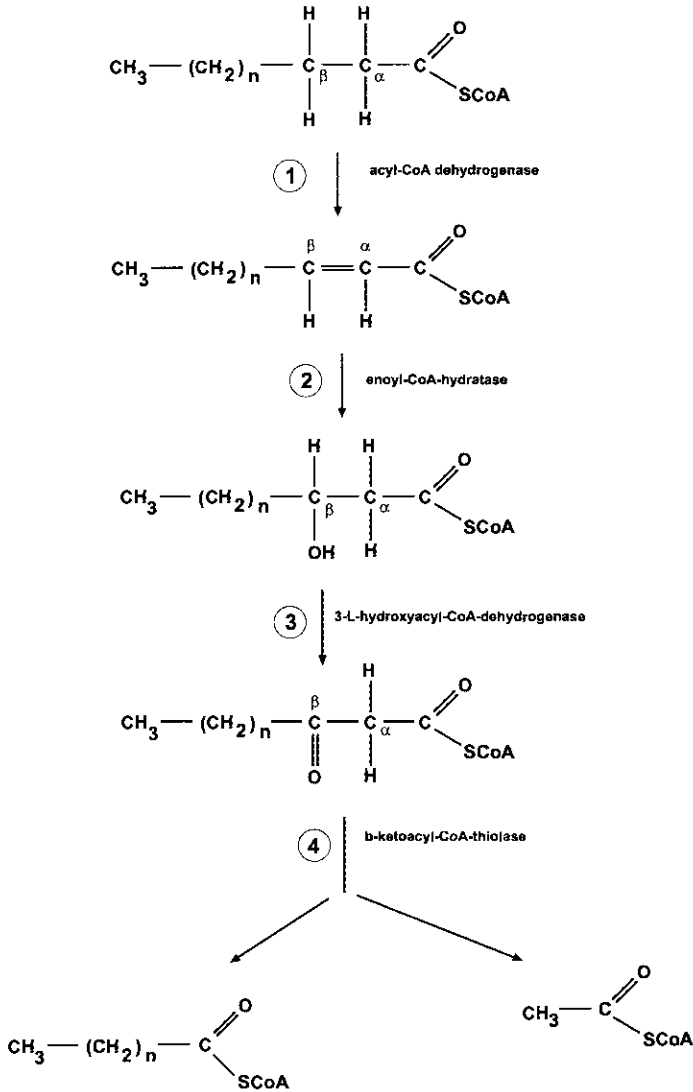


The FAD is a cofactor of acyl-CoA dehydrogenase, and the  $\text{FADH}_2$  is reoxidized via two proteins, the electron transfer flavoprotein (ETF) and the ETF:ubiquinone oxidoreductase transferring electrons to ubiquinone (to yield  $\text{QH}_2$ ), which is reoxidized via the electron transfer chain. Superficially the acyl-CoA dehydrogenase therefore resembles succinate dehydrogenase in the use of flavin as the immediate acceptor of hydrogen, followed by electron transfers via iron-sulfur centers to ubiquinone.





**Figure 6.2** **A:** Trans-esterification between acyl-CoA and carnitine. In the cytosol the acyl group is transferred from acyl-CoA to carnitine. In the mitochondrial matrix the reaction is reversed. **B:** A permease in the mitochondria is responsible for the shuttling of acyl groups into mitochondria.

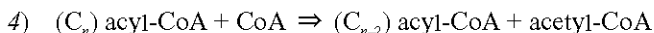


**Figure 6.3** The essential steps in  $\beta$ -oxidation of fatty acids in mitochondria. Four enzymatic reactions are required to reduce the acyl moiety of acyl-CoA by two carbons, with the formation of one molecule of acetyl-CoA. The series of reactions are repeated until the acyl moiety is completely converted to acetyl-CoA. Complications arise when the acyl group already contains double bonds, or if the number of carbons is uneven (see text).

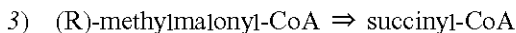
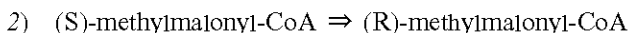
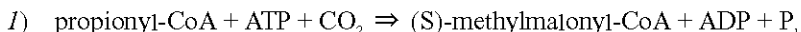
This hydration reaction resembles the formation of malate from fumarate and is catalyzed by enoyl-CoA hydratase. It is followed by an oxidation to  $\beta$ -ketoacyl-CoA



In the final step, another CoA is a second substrate, and the reaction splits off a two-carbon unit to form acetyl-CoA, leaving acyl-CoA, but now two carbon atoms shorter than at the start:



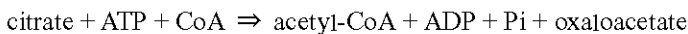
The series of reactions 1–4 is repeated, and a saturated fatty acid with an even number of carbons can be converted to acetyl-CoA with no further complications, except that different acyl-CoA dehydrogenases are used as the carbon chain of the acyl group gets shorter. Unsaturated fatty acids can be dealt with by a combination of isomerases and reductases (requiring NADPH), depending on the position of the double bonds and their configuration. The  $\beta$ -oxidation of odd-chain fatty acids leads to the formation of propionyl-CoA, which must be dealt with by a distinct series of reactions:



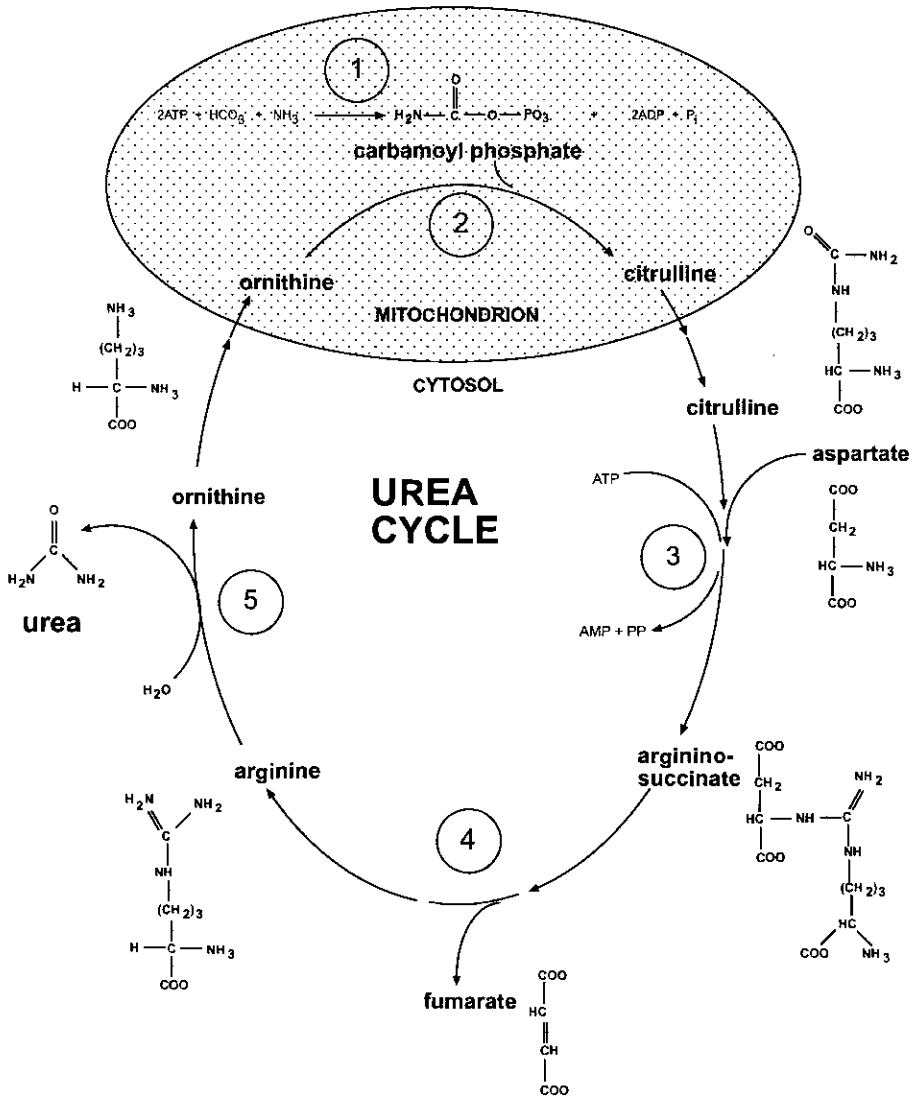
Reactions 1–3 are catalyzed by a carboxylase, a racemase, and a mutase, respectively. The methyl-malonyl-CoA mutase is an unusual enzyme requiring 5-deoxyadenosylcobalamin (coenzyme  $B_{12}$ ) as a cofactor; this cofactor contains Co(III) in the center of a ring resembling heme, and has the only carbon–metal bond known in biology. Vitamin  $B_{12}$  deficiency was first shown in 1926 to cause pernicious anemia, and humans must acquire it from their food (meat). Uptake and transport in the bloodstream requires specific proteins, cobalamins. The solution of the crystal structure of cobalamin by D. Hodgkin was a notable achievement in crystallography in 1956, which was later dwarfed only by the determination of the crystal structure of whole proteins.

While the acetyl-CoA formed during  $\beta$ -oxidation could enter the Krebs cycle as expected, its actual fate in liver is different. A process referred to as ketogenesis converts acetyl-CoA into compounds called ketone bodies which include acetone, acetoacetate and  $\beta$ -hydroxybutyrate. The solubility of ketone bodies makes them readily transportable by the blood to other organs and tissues where they serve as alternate metabolic fuels. For example, when fats are mobilized during starvation, ketone bodies can substitute for glucose in the brain.

Although the biosynthesis of fatty acids takes place in the cytosol, and its details get short shrift in this book on mitochondria, it is appropriate and relevant to discuss one of the first steps required for this cytosolic sequence to take place: acetyl-CoA has to get out of the mitochondria. This is achieved by making citrate in the mitochondria, which can be exported by a transport system for tricarboxylic acids. In the cytosol acetyl-CoA is regenerated by ATP-citrate lyase







**Figure 6.4** The urea cycle. The reactions of the urea cycle are distributed between mitochondria and the cytosol. The synthesis of carbamoyl phosphate and its condensation with ornithine to form citrulline take place in the mitochondrial matrix. The remaining reactions occur in the cytosol.

## 6.4 THE UREA CYCLE

The Krebs cycle keto acids  $\alpha$ -ketoglutarate and oxaloacetate can be withdrawn to form amino acids by transamination or added to the cycle by the easily reversible reaction, and it may be worth mentioning that mammalian cells in tissue culture thrive in media with added glutamine, even though it could be considered a nonessential

amino acid. It enters the Krebs cycle as  $\alpha$ -ketoglutarate, thus providing nitrogen in a transamination reaction. This nitrogen is recaptured when oxaloacetate is converted to aspartate (and asparagine). In fibroblasts these two nonessential amino acids are derived almost exclusively from glutamine via the operation of the Krebs cycle [4,5].

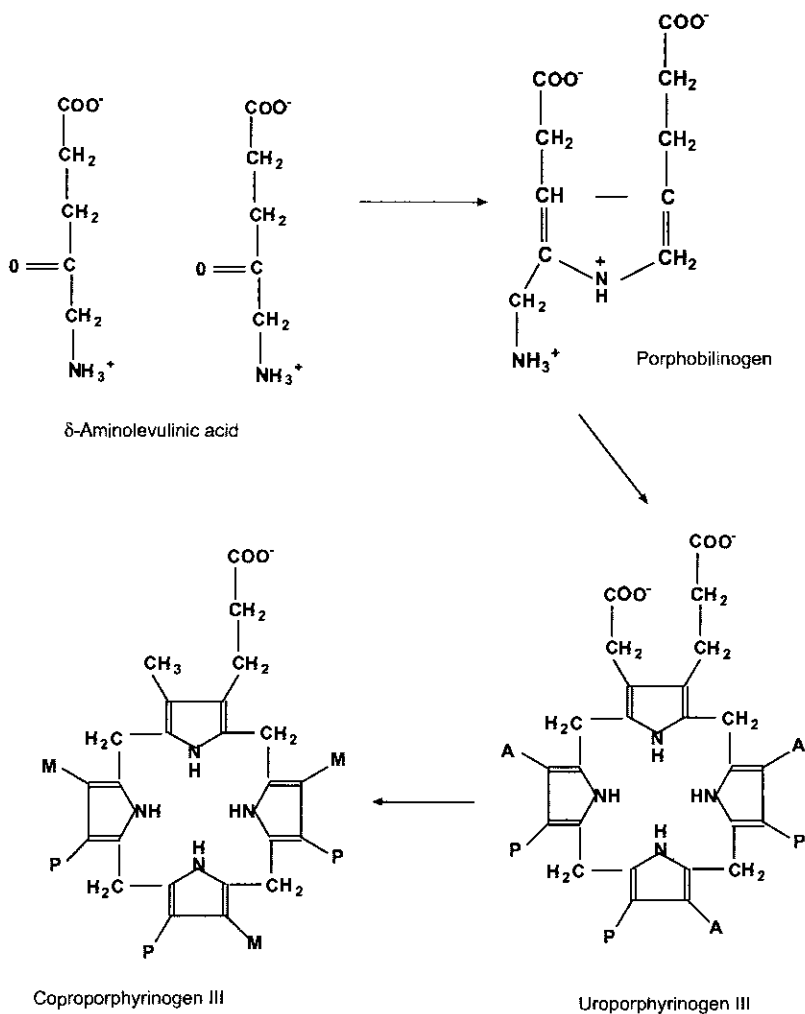
A most significant role of mitochondria in amino acid metabolism is the participation in the urea cycle. The urea cycle was the first metabolic cycle discovered and described by Krebs five years before his more famous citric acid cycle. The cycle is crucial for the breakdown of excess amino acids and the conversion of excess nitrogen into urea, the excretable form of nitrogen in many terrestrial animals. The major activity takes place in the liver, from where urea is delivered via the bloodstream to the kidneys for excretion in urine.

As expected, there is one reaction creating a precursor, carbamoyl phosphate, which can enter the cycle by condensation with a cycle intermediate, and products including urea are generated in various steps in the cycle. Only some reactions of the cycle take place in mitochondria; it is completed in the cytosol. The complete cycle is shown in Figure 6.4. The two reactions taking place in mitochondria are the formation of carbamoyl phosphate from ammonia and bicarbonate, driven by the hydrolysis of ATP, and the condensation of carbamoyl phosphate with ornithine to form citrulline. Carbamoyl phosphate synthetase I, the enzyme required for step 1 is allosterically regulated by *N*-acetylglutamate, made from glutamate and acetyl-CoA. Increased glutamate concentrations signal excess amino acids (nitrogen), and an increase in *N*-acetylglutamate activates carbamoyl phosphate synthesis.

## 6.5 BIOSYNTHESIS OF HEME

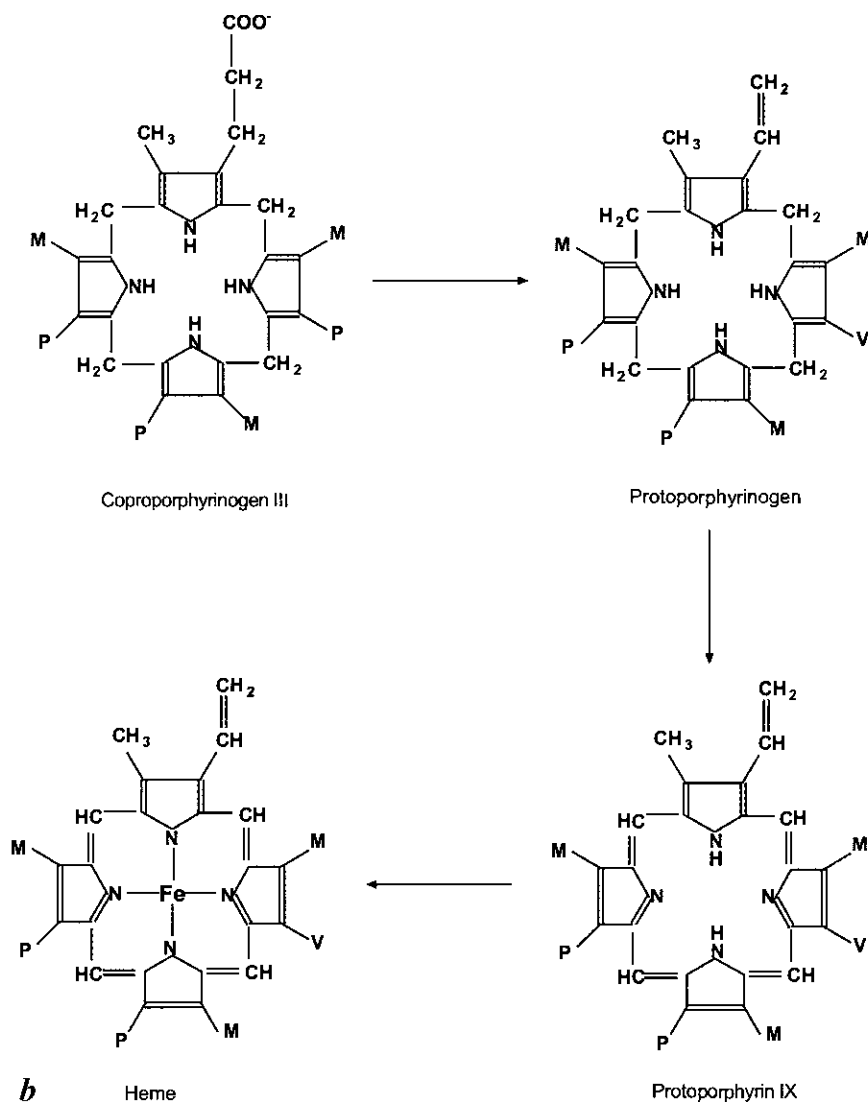
Studies on respiration, cytochromes and heme leading to the elucidation of the role of mitochondria have been intricately linked for over a century. In hemoglobin in the blood the heme group is used to bind and transport oxygen. In the electron transfer chain heme groups associated with peptides form the cytochromes. Another important cytochrome is the cytochrome P450, an enzyme system used primarily in the liver for metabolizing various toxic compounds such as carcinogens. An intermediate in heme biosynthesis is a tetrapyrrole derivative (see below), a conjugated, planar ring structure without the metal ion, and in plants this intermediate is the precursor for chlorophyll.

The use of isotope tracers made it possible in the late 1940s to investigate the precursors for heme biosynthesis, and the whole pathway was established in the following decade (Figure 6.5). It starts in the mitochondria with a Krebs cycle intermediate, succinyl-CoA, and the formation of  $\delta$ -aminolevulinic acid (ALA) involving the rate-limiting enzyme ALA synthase. It was described earlier how ALA protein levels can be controlled by regulating the efficiency with which ALA mRNA is translated (see also below). ALA leaves the mitochondria to be converted to coproporphyrinogen III in a complex series of reactions. An oxidative modification of the ring structure is coupled to its transport back into mitochondria, where the synthesis of heme is completed with another oxidation and the final addition of the iron to the center of the



porphyrin ring. It is noteworthy that the latter reactions require molecular oxygen and thus are sensitive to oxygen concentrations.

ALA synthase is the key regulatory enzyme in the liver, but may not play this role in immature erythrocytes, another tissue in which heme biosynthesis is prominent. Under these conditions ALA synthase may be feedback inhibited by its product. Its import into mitochondria may be controlled. It is also among the group of proteins associated with iron transport, storage, and metabolism that are encoded by mRNAs



containing iron-responsive elements (IRE) in their untranslated regions. When iron is deficient, the cytosolic aconitase is in an open conformation (the iron-sulfur center is dissociated) and capable of blocking the translation of ferritin and ALA mRNAs by binding tightly to the IRE. This mechanism allows the biosynthesis of heme to be adjusted to the availability of iron (Fe(II)) in the cell and mitochondria.

As described in an earlier chapter, the expression of genes encoding mitochondrial proteins in yeast is sensitive to oxygen. More specifically, the need for molecular oxygen in the final oxidations in the pathway makes heme synthesis sensitive to oxygen.

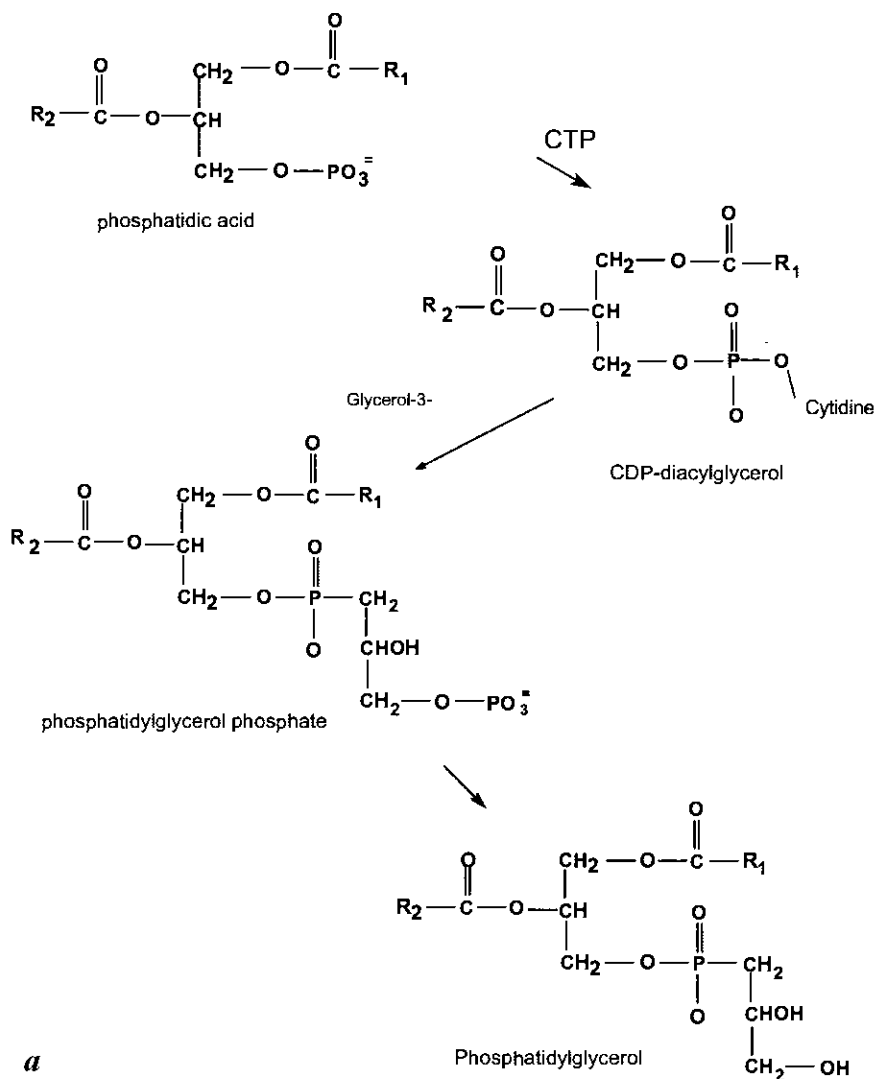


## 6.6 CARDIOLIPIN AND LIPID BIOSYNTHESIS/METABOLISM

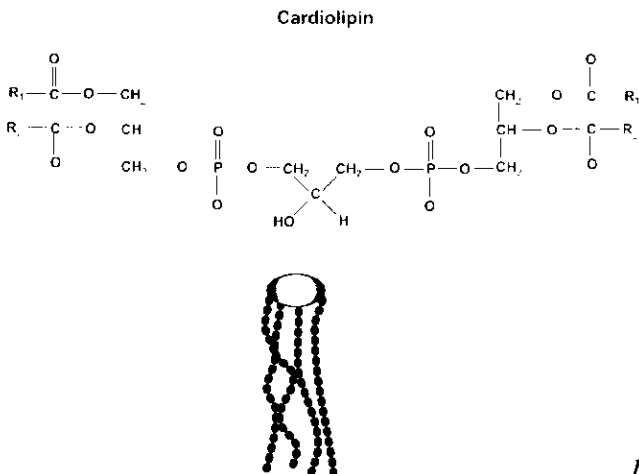
A comprehensive review of the lipid composition of mitochondria has been published by Daum [7]. It is generally agreed that the inner membrane has one of the highest protein:lipid ratios of all biological membranes studied, but in spite of the dense packing of protein complexes and the various carriers and import machinery, the membrane nevertheless behaves as a fluid mosaic [8,9]. One of the most noteworthy aspects of the inner mitochondrial membrane is that it is the exclusive location for the lipid diphosphatidylglycerol, also named cardiolipin. This lipid has long been believed to be absolutely essential for the functioning of various complexes and integral membrane proteins, but some doubts are now being raised from the study of a mutant yeast strain (see below). Purified, functional preparations of such complexes have inevitably contained cardiolipin, suggesting that this lipid has a special affinity for such proteins. More discussion on the need of this lipid for proper function of these complexes is found in Chapter 5. The proposed functions of this unique lipid have also been broadened to include a participation in mitochondrial protein import, protein folding and assembly, that is, in the assembly and maintenance of the inner membrane. However, most of these roles of cardiolipin must remain highly speculative. In this discussion the focus will be on the synthesis of this lipid and some other characteristics of its composition. A recent report [11] describes a yeast mutant with the cardiolipin synthase gene, *CLS1*, knocked out, and no cardiolipin found in any of the membranes. It may have been a surprise to find the cells viable in both fermentable and nonfermentable carbon sources.

Cardiolipin is found in the membranes of eubacteria such as *Escherichia coli*, and in the mitochondrial membranes of all eukaryotes studied so far, but not in archaeobacteria [11–13]. It is an unusual phospholipid with four acyl chains. The synthesis in bacteria involves the combination of two molecules of phosphatidyl glycerol with the elimination of one glycerol molecule. By contrast, in eukaryotes (liver, *Saccharomyces cerevisiae*, *Neurospora crassa*, bean sprouts, and mussels) the two starting substrates have been shown to be phosphatidylglycerol (PG) and CDP-diacylglycerol ([10,13] for reviews and listing of additional references). The phosphatidyl group from CDP-diacylglycerol is transferred to PG by the enzyme cardiolipin synthase (Figure 6.6). The evolution of the reaction in eukaryotes from the biosynthetic pathway in bacteria has been postulated to be a consequence of the different levels of the precursor present in bacteria and mitochondria [10]. In the former, phosphatidyl glycerol is abundant, and a simple, reversible trans-esterification can be driven toward cardiolipin by substrate availability. In mitochondria the precursor may be limited, and the forward reaction is driven by the irreversible cleavage of a high-energy anhydride bond in phosphatidyl-CMP (CDP-diacylglycerol). Hence the mitochondrial reaction is highly exergonic.

Properties of cardiolipin synthase from various mitochondrial preparations have been described [10,13,15]. Although it had long been suspected to be found exclusively in mitochondria, a detailed study has localized it to the matrix side of the inner membrane in rat liver mitochondria [16], that is, it is an integral membrane protein in this membrane with a large hydrophilic domain exposed to the matrix. The enzyme



has been solubilized with the help of detergents and purified to a significant degree from yeast and mammalian sources. Several of the properties of this enzyme appear to be similar in all eukaryotes examined, in support of the monophyletic origin of mitochondria [13], but there are also some distinctions between the yeast enzyme and the mammalian enzyme of as yet uncertain significance. Most notably, the molecular mass of the yeast enzyme has been reported to be 20–30 kDa, in contrast to a molecular mass of around 50 kDa for the mammalian enzyme.



**Figure 6.6** A: Outline of the reactions and major intermediates involved in cardiolipin biosynthesis. B: Structure of cardiolipin and a predicted 3-dimensional model of the molecule.

Schlame and colleagues [10,13,15] also addressed the problem of the molecular acyl species found in cardiolipin of various species. In all organisms a high percentage of symmetrical acyl species was found, that is, species with identical diacylglycerol moieties. Acyl chains were either identical or differed only by two carbons or one double bond, respectively. Minimum energy conformations were calculated by these authors, but they point out that the conformation in a real membrane and in association with protein complexes may be quite different. Not surprisingly, the models suggest all acyl chains to be arranged more or less in parallel, and in a highly cartoonlike fashion one could represent cardiolipin as a single polar head group with four aliphatic chains (Figure 6.6B). Another notable property of cardiolipin is that it is an anionic phospholipid.

Since cardiolipin is synthesized on the inside of the inner membrane and is therefore most likely found in the inner leaflet of the bilayer, one has to ask whether it can be distributed beyond this particular location. A "flippase" would be required to translocate it to the outer leaflet. On the other hand, in the immediate vicinity of the multisubunit integral membrane protein complexes where cardiolipin is found the distinction between outer and inner leaflets may be meaningless. Movement to the outer membrane is even less likely but still not definitely excluded by experiments. Since mitochondria may fuse with each other, but not (normally) with other membranous



organelles in a cell, the exclusive presence of cardiolipin in mitochondria can be explained.

The problem is less settled for the precursor phosphatidylglycerol (PG). It is derived from the precursor phosphatidylglycerophosphate (PGP) (catalyzed by a phosphatase). PGP is made from CDP-diacylglycerol, and PGP synthase has also been localized in mitochondria. Such a finding would explain the presence of cardiolipin and PG in mitochondria, but PG appears to be a minor constituent of other intracellular membranes of mammalian cells, and it may be necessary to postulate a second, cytoplasmic isozyme for PGP synthase [17].

The biosynthesis of the other common phospholipids found in mitochondria is not exclusive, and detailed reviews on phospholipid metabolism are beyond the scope of this treatise [7,12,14]. If the synthesis of these lipids takes place in the smooth endoplasmic reticulum, a mechanism must be found for distributing these lipids to other cellular locations. Vesicular traffic from the ER via the Golgi to the plasma membrane, and vesicular traffic in both directions between these membranes and various intermediate compartments can be used to rationalize the distribution of lipids within a cell. Nevertheless, mechanisms must be postulated to explain the nonuniform distribution of lipids such as cholesterol in these membranes. Less attention appears to have been devoted to the delivery of lipids to mitochondria: are specialized shuttle vesicles involved? Are lipids delivered individually by means of specialized carrier proteins? "Confluencies" between the outer mitochondrial membrane and the endoplasmic reticulum have been observed [reviewed in Reference 18]. It is not clear whether it is a transient state, and whether it serves to import components into mitochondria, or whether it represents an early step in degradation. When mitochondria are vitally stained with the cyanine dye DiOC<sub>6</sub>, the dye is first accumulated by mitochondria, but subsequently it can be observed in the ER after the mitochondria have lost their elongated shape [18].

A few generalizations and basic principles are expertly reviewed by Daum with special reference to mitochondria [7], and more recently by Kent [19] and Dowhan [14] from a more general perspective. While the lipid content of mitochondria is somewhat variable as expected, differing between tissues as well as between organisms, it is clear that the major phospholipids of mitochondria in addition to cardiolipin (10–20%) are phosphatidylethanolamine, PE (20–40%) and phosphatidylcholine, PC (35–50%). The other phospholipids are present at a level generally less than 5%, and cholesterol is present only in traces. These generalizations apply to vertebrate tissues as well as a variety of microorganisms, and plant mitochondria. The latter also contain a variety of free and derivatized sterols [see Reference 7 for review and original references]. A further distinction can be made between the outer and inner membranes of mitochondria, and a few attempts have been made to characterize the differences. The presence of cardiolipin in the outer membrane is still controversial, since contamination is a possibility. Other differences in the ratio of PE:PC, or the relative amounts of phosphatidylinositol (PI), have been observed, but the functional significance is still obscure.

The origin and site of synthesis of mitochondrial lipids are areas still under investigation and in a state of evolution [20]. Conclusions are somewhat subject to the suc-

cess of fractionation schemes, most notably, fractionation of mitochondria from microsomes and other vesicular structures, identified by marker enzymes. Phosphatidylethanolamine is made in mitochondria from the decarboxylation of phosphatidylserine (PS). A search for the cellular location (in yeast) of phosphatidylserine synthase has identified a microsomal subfraction, but the absence of typical microsomal marker enzymes has led Zinser and colleagues [19] to propose a novel particle population. One is forced to conclude that PS is imported into mitochondria, but some of the PE made must also be exported to other cellular membranes where it is found. Alternatively, a second PS decarboxylase may exist in a cytosolic membrane, and indications for its existence are found from the study of a yeast mutant defective in the mitochondrial PS decarboxylase [see Reference 18 for discussion].

While cell fractionation studies have proved their worth over the decades, the fractionation of subcellular membranous structures has been a technical challenge due to microheterogeneity, and results are subject to misinterpretation when contamination cannot be ruled out. In the future one can expect that model organisms like yeast will allow systematic gene disruptions to explore how the absence of a particular enzyme (and hence the product from this pathway) affects growth and other cellular activities. An example of the unexpected type of result derived from a genetic approach is the finding that unsaturated fatty acids are essential for the distribution of mitochondria to the daughter cells in the yeast *Saccharomyces cerevisiae*, presumably because the necessary shape changes (elongation) are dependent on the fluidity of the mitochondrial membranes [21].

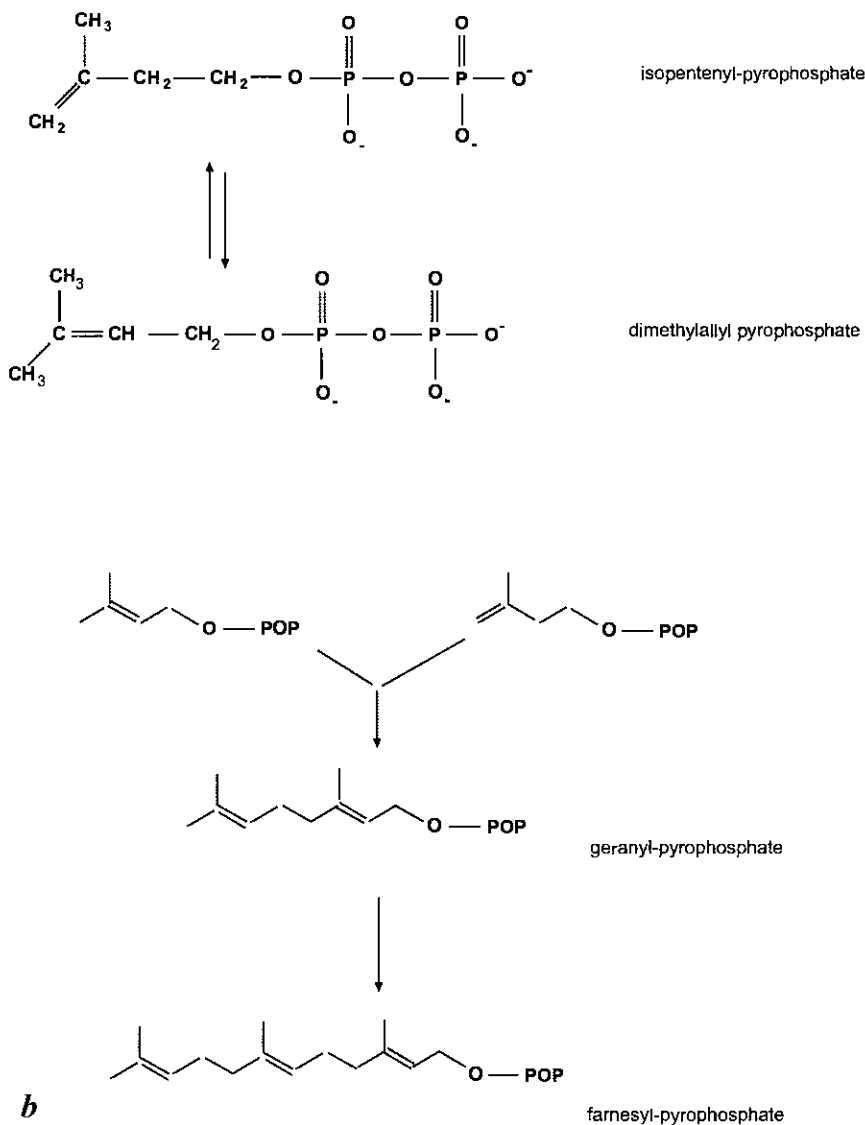
## 6.7 BIOSYNTHESIS OF UBIQUINOL (COENZYME Q)

The biosynthesis of ubiquinol was elucidated some time ago from studies in *Escherichia coli* and yeast *Saccharomyces cerevisiae*. Genetic studies were critical, since CoQ deficient strains of each organism could be isolated, and intermediates accumulating at different stages in the biosynthetic pathway gave clues about the sequence of reactions. Although the pathways differ slightly in some intermediate steps in prokaryotes and yeast, eight genes have been defined in each organism by the isolation of eight complementation groups (*coq1-coq8* in yeast). The CoQ-deficient yeast strains are respiration-deficient and fail to grow on nonfermentable carbon sources, but do grow on glucose [22]. When cytochrome c oxidase is assayed in vitro from such strains, the missing activity can be restored by the addition of CoQ.

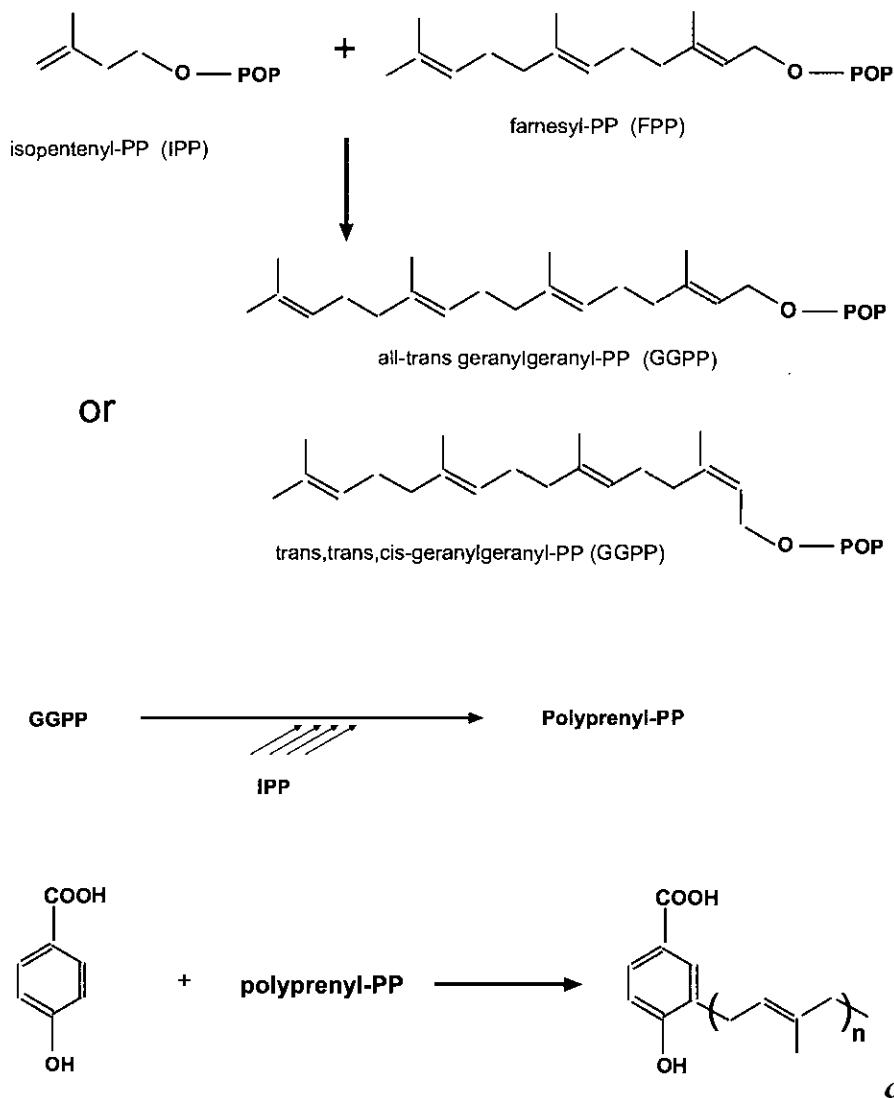
The reactions defined by mutations in CoQ/ubiquinone-deficient bacteria or yeast are the final reactions unique for the biosynthesis of ubiquinone. Key starting materials in this pathway are *p*-hydroxybenzoic acid and polyisoprene diphosphate. The final polyprenyl tail contains *n* isoprene units, where *n* varies between organisms. In mammalian mitochondria *n* = 10, and hence the compound is also referred to as Q<sub>10</sub>. The Q<sub>10</sub> derivative is extremely insoluble in water, and in in vitro experiments as well as in therapeutic applications derivatives with shorter tails are often used.

The synthesis of the polyprene tail begins in the well known pathway from acetyl-CoA via hydroxymethylglutaryl-CoA, mevalonate, geranyl-pyrophosphate and farnesyl-

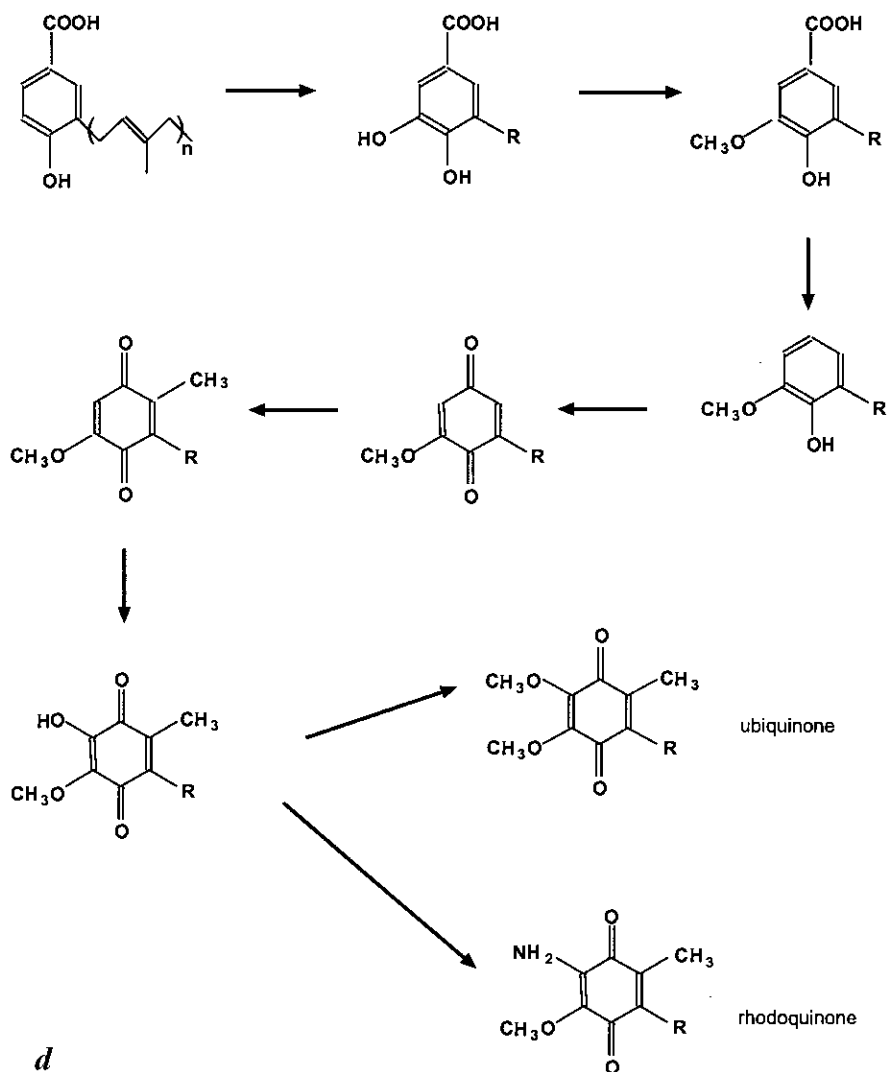




boxylation yield isopentenyl pyrophosphate (IPP), which is reversibly isomerized to dimethylallyl pyrophosphate (DMA-PP). A condensation of IPP and DMA-PP yields geranyl pyrophosphate (GPP), and the addition of another isopentene unit leads to farnesyl pyrophosphate (FPP). The enzymes are soluble cytosolic enzymes, with one exception, the enzyme HMG-CoA reductase being an integral membrane protein in the endoplasmic reticulum. It has received a considerable amount of attention as a key regulatory enzyme in cholesterol biosynthesis. It may therefore also influence the rate



of synthesis of the other products at the end of the various branches, but while its regulatory role in cholesterol biosynthesis is undisputed, modulating its activity by dietary conditions or inhibitors has been found to have no significant influence on the biosynthesis of dolicho1, ubiquinone, or the isoprenylation of proteins. This apparent dilemma has been addressed by several authors, and a plausible model referred to as the “flow diversion hypothesis” has been postulated. According to this hypothesis, most of the enzymes involved in the branch-point reactions have a high affinity for FPP, and flux into these branches can occur even at low FPP concentrations. The branch-point enzyme squalene synthase, on the other hand, has a low affinity for FPP,



**Figure 6.7** Biosynthesis of ubiquinone. **A** and **B**: Reactions leading to the biosynthesis of farnesyl-pyrophosphate. Isopentenyl-pyrophosphate and dimethylallyl-pyrophosphate are readily isomerized, and two different isomers condense to form geranyl-pyrophosphate. See text for a more detailed discussion. **C**: The precursor farnesyl-pyrophosphate can be elongated by the addition of the isopentenyl-unit to form GGPP (two isomers shown, of which the all trans isomer is the important one for the synthesis of ubiquinone). Further additions yield polyprenyl-pyrophosphate of various lengths (see text). The condensation of hydroxybenzoic acid with polyprenyl-PP yields a distinct precursor for ubiquinone synthesis. **D**: Several modifications on the ring structure include hydroxylations, decarboxylation, *O*-methylation and methylation. Near the end of this series of reactions another branch point has been proposed [26,27], which can lead to the formation of ubiquinone or the closely related rhodoquinone (see text).

and thus cholesterol biosynthesis will occur only when high concentrations of FPP are achieved by an activation of HMG-CoA reductase.

Protein isoprenylation with FPP or GGPP (Figure 6.7C) as isoprenoid donors is now recognized to be widespread in eukaryotic cells. Proteins such as the ras protein can be derivatized with long hydrophobic prenyl side chains which serve to anchor them on membranes at specific subcellular locations. A full discussion of this subject is beyond the scope of this chapter. As shown in Figure 6.7C, geranyl-geranylpyrophosphate (GGPP) is made from FPP and IPP by enzymes named GGPP synthases (a subset of prenyltransferases). There are at least two types, and the product can be either the *all-trans*-GGPP, or the *trans, trans, cis*-GGPP. These enzymes differ in their intracellular distribution; they may be cytosolic or membrane-associated; they have been found in mitochondria, peroxisomes and the ER; either  $Zn^{2+}$  or  $Mg^{2+}$  may be absolutely required. The cytosolic enzyme appears to produce only *all-trans*-GGPP, while *trans, trans, cis*-GGPP is produced by membrane-bound enzymes.

Additional prenyltransferase reactions with IPP as donor serve to elongate the chain, but the *all trans*- and the *trans, trans, cis*-GGPP have different fates. The latter is used for the synthesis of dolichol, containing between 16 and 23 isoprenoid units in animal cells. Dolichol is phosphorylated and esterified to oligosaccharides to form an essential intermediate in glycoprotein syntheses on the ER. This is another long story not to be pursued here. The *all trans*-GGPP has been clearly established as the substrate for protein isoprenylation, and it is also likely to be the substrate for trans-prenyltransferase leading to the synthesis of *all trans*-polyisoprenoid-PP. Consisting of about 9 or 10 units, the polyisoprenyl group can be transferred to p-hydroxybenzoic acid in the first distinct step leading to ubiquinone (Figures 6.7C,D).

The subcellular localization of the distinct prenyl transferase enzymes could be used to control the destination of the various products, and also serve in establishing separate regulatory mechanisms. Cell fractionation studies in conjunction with genetic analyses are not yet complete to present a final picture. As long as ubiquinone was thought to be exclusively present in the inner mitochondrial membrane, the presence of the biosynthetic enzymes in mitochondria could be rationalized. However, it has since been established that ubiquinone is found in extramitochondrial membranes as well. Are there multiple enzymes for ubiquinone synthesis, or can this molecule be exported from mitochondria? The more abundant species is found to be the reduced form (ubiquinol), and there appears to be agreement that ubiquinol has an important function as an antioxidant [24]. Its abundance exceeds  $\alpha$ -tocopherol (vitamin E) by a factor 10 in animal cells.

An interesting problem presents itself in certain eukaryotic microorganisms which have complex life cycles and may include a stage which exists in an aerobic environment (carrying out normal respiration and oxidative phosphorylation), and another stage living in an environment requiring drastic adjustments in metabolism and even anaerobic electron transfer [25]. One such example of a parasite, the liver fluke, *Fasciola hepatica*, has been studied in some detail. The free-living stage (aerobic) oxidizes succinate and transfers electrons via  $UQ_{10}$  to complex III of the electron transfer chain. The parasitic stage oxidizes NADH via complex I and the reduction of fumarate to succinate by fumarate reductase. The mobile carrier between complex I and

FRD is another quinone, rhodoquinone (RQ) [26,27]. Experiments with [2-<sup>14</sup>C]-mevalonate demonstrated that UQ and RQ can be synthesized de novo in these organisms. Rhodoquinone has an amino group substituted on the quinone ring, and this modification must be part of the developmental program of this organism. The substitution alters the redox potential of the RQ/RQH<sub>2</sub> redox couple which appears necessary to allow electron flow to occur in the reverse direction in FRD compared to complex II [28].

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# 7

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## MITOCHONDRIAL MUTATIONS AND DISEASE

Previous chapters have made frequent reference to nuclear and mitochondrial mutations that have had global effects on mitochondrial biogenesis. Mutations affecting DNA replication and transcription, RNA processing, and translation of mitochondrial mRNAs, as well as specifically nuclear mutations that cause defects in the protein import machinery of mitochondria were discussed in the context of the mechanisms that operate in mitochondrial proliferation and the assembly of the mitochondrial electron transport chain. The mutations were of interest because they illuminated the role of specific gene products in the biogenesis of mitochondria. This chapter will emphasize effects of mitochondrial dysfunction on the cell or on the whole organism. Many in-born errors of metabolism are due to nuclear mutations and hence enzyme deficiencies in metabolic reactions taking place in the mitochondrial matrix. As an example, one can mention ornithine transcarbamylase deficiency, one of several defects causing disorders in the urea cycle. A discussion of such metabolic diseases is not intended here. Instead, the focus will be on mutations in the mtDNA affecting the capacity for oxidative phosphorylation and hence one of the major functions of the mitochondria as the powerhouse of the cell. It should be stated at the outset that nuclear mutations can also have a direct effect on oxidative phosphorylation, since both genomes contribute to the complexes of the mitochondrial electron transport chain. Severe defects (null mutations) are likely to be lethal in complex organisms, but missense mutations causing partial deficiencies are to be expected. While the rules of mendelian inheritance will apply to such nuclear mutations, mitochondrial mutations will be passed along the maternal lineage. Additional complications arising from variations in heteroplasmy will be elaborated below.

The existence of mtDNA mutations was first recognized in cell culture with lower eukaryotes, such as yeast, and to a very limited extent with mammalian cells in cul-

ture. During the past decade a new field of “mitochondrial medicine” has been created from the discovery that a complex and variable set of symptoms in humans could be caused by partial deficiencies in oxidative phosphorylation due to mitochondrial mutations. The nature of mitochondrial diseases (often associated with neurological symptoms among others, frequently characterized by a delayed onset) has stimulated speculations that mitochondrial mutations might be implicated in other well-known neurodegenerative diseases such as parkinsonism and Alzheimer’s disease. In fact, the general phenomenon of aging has attracted attention from the point of view of an accumulation of mitochondrial mutations being a cause for a deterioration of OXPHOS capacity and hence a deterioration of diverse functions.

Apoptosis is a normal phenomenon in development, and it is the failure of apoptosis at appropriate times or stages that is believed to play a role in tumorigenesis. A number of diverse investigations have suggested a possible role of mitochondria in the apoptotic pathway. An examination of the evidence is included in this chapter, but the final word probably cannot be spoken in this rapidly advancing field.

Finally, this chapter includes a summary and discussion of the salient features of fungal senescence and male sterility in plants because mitochondria and mitochondrial genomes (and mitochondrial plasmids) are strong genetic determinants in the expression of these phenomena.

## 7.1 IN CELL CULTURE

### 7.1.1 Mitochondrial Mutations in Microorganisms

For very familiar practical and theoretical reasons the budding yeast, *Saccharomyces cerevisiae*, has become one of the most widely used model systems in which to study eukaryotic molecular genetics, cell biology, and even cell physiology. Growth requirements are minimal, and growth will occur under a wide variety of conditions. The yeast cell has all or most of the typical subcellular organelles and compartments of a eukaryotic cell. Its genome is relatively small, and the complete sequences of all of its chromosomes have recently become available. Large numbers of cells can easily be cultured in suspension or on plates. Mutant selections on a relatively large scale are possible, and clones are readily isolated. A system of mating and sexual reproduction has made the standard genetic methodology applicable for mapping and complementation analysis. Most readers will not need to be reminded of the enormous advantages of yeast as a model system, and it may at times be more interesting to ask, What biological problems cannot be tackled with the help of yeast? Even prions appear to be found in yeast [1].

Studies on plant variants early in this century had noted nonmendelian (maternal) types of inheritance, a phenomenon that became known as cytoplasmic inheritance [2,3]. Mutations in chloroplast DNA were eventually demonstrated to be responsible for these original observations. The field received a major boost when cytoplasmic inheritance was discovered in yeast [4], and a large variety of yeast mutants classified as *pet* mutants, *mit* mutants,  $\rho^-$  or  $\rho^0$  mutants has been useful in making pioneering dis-

coveries on mitochondrial inheritance, mitochondrial functions, oxidative phosphorylation, and so on. A detailed description of all of these would constitute a book all by itself [5]. Only some general principles and insights will be discussed in the following section and illustrated with some specific examples.

A property of yeast cells is that they can grow under a variety of different conditions. Such conditions are likely to be encountered in the wild as well, and the ability of yeast to adapt to different defined conditions in the laboratory continues to amaze and to provide systems for the study of gene regulation in many laboratories. Of primary significance for the present discussion is the ability to adapt to the carbon source in the medium, and to the availability of oxygen. The expression of multiple genes is adjusted to prevailing conditions. When a fermentable carbon source and oxygen are present, many genes involved in transport, metabolism of other sugars, and, most significantly, the biosynthesis of the entire electron transport chain and ATP synthase are repressed. This phenomenon, referred to as "glucose repression," has been the subject of numerous reviews [6–10]. In a previous chapter on nuclear mitochondrial interactions many aspects of this phenomenon relevant to mitochondrial biogenesis have already been presented.

Here it is simply relevant to emphasize that wild-type yeast cells can grow on media either rich in glucose (a fermentable carbon source) or deficient in glucose (in the presence of nonfermentable carbon sources such as acetate, glycerol, or ethanol). In combination with replica plating, it is therefore straightforward to isolate mutant clones that can grow on glucose but are unable to grow in the presence of glycerol alone. Among the possible mutants, respiration-deficient mutants (*res<sup>-</sup>*) form a large group, and, because they tend to form small (petite) colonies under defined conditions of glucose deprivation, they are routinely referred to as *pet* mutants. Collectively they have been expertly described and reviewed by Dujon [5] and Tzagoloff and Dieckmann [11]. Mutations giving rise to this phenotype can be subdivided into nuclear and mitochondrial mutations. Nuclear mutations are local and generally affect single, specific genes whose function has been exemplified in numerous instances in earlier chapters of this book. The mitochondrial mutations can again be subdivided into point mutations affecting single functions (referred to as *mit<sup>-</sup>*) and large deletions of mtDNA (termed cytoplasmic petites or *rho<sup>-</sup>* or  $\rho^-$ ) [12]. Mutants completely lacking mtDNA are termed *rho<sup>0</sup>* ( $\rho^0$ ) strains, and they can be obtained by prolonged culture of yeast cells in glucose and sublethal concentrations of ethidium bromide. Since the deletions in *rho<sup>-</sup>* mutants include tRNA genes most of the time, they are incapable of mitochondrial protein synthesis and thus lack the entire electron transport chain. When *mit<sup>-</sup>* mutants are crossed with *rho<sup>-</sup>* mutants, marker rescue by recombination can occur, if the *mit* mutation is in a region of the genome still present as a wild-type sequence in the *rho<sup>-</sup>* mitochondria. Respiration-competent diploid cells arise at high frequency by this mechanism, which involves recombination rather than complementation. A recombination event is necessary to generate wild-type diploid cells because it is found that "merodiploid" or heteroplasmic mitochondria are not maintained under routine culture conditions. Instead, rapid mitotic segregation of mitochondrial genomes takes place, resulting in a loss of potentially complementing sequences [12].

A particularly useful new method in yeast mitochondrial genetics has been the development of the transformation of mitochondria by high-velocity bombardment of yeast cells with tungsten microprojectiles to which cloned DNA sequences are adsorbed. By this method, DNA constructs made *in vitro* can be introduced into mitochondria of *rho*<sup>0</sup> strains to form “synthetic *rho*” strains. After mating with *rho*<sup>+</sup> strains the DNA constructs can recombine with the mtDNA and deliberate mutations can be introduced into mtDNA. Details of the procedure can be found in the original publications and authoritative reviews [12,13].

### 7.1.2 Respiration-Deficient Mammalian Cells

Twenty years ago it would probably have been considered impossible to obtain mammalian cell mutants in tissue culture with severe defects in respiration. Attempts to grow mammalian cells under anaerobic conditions had consistently failed, and since normally such cells would not have experienced severe anaerobiosis, it was believed that oxidative phosphorylation was an essential activity in the energy metabolism of such cells. There had been a great deal of speculation about an altered energy metabolism in cancer vs normal cells since the days when O. Warburg [14] had proposed that tumor cells had partially reverted to a more “primitive” type of energy metabolism in which glycolysis played a larger role, but no specific enzyme or activity had been shown to be affected in cancer cells, which would have explained the elevated secretion of lactate by cells grown under normal aerobic conditions. An informative, entertaining, and highly personal review of the literature on this subject up to about 1975 has been written by Racker [15].

Serendipity played a large role in the isolation and characterization of the first respiration-deficient Chinese hamster fibroblast mutant cells in tissue culture [16–18]. The original mutant cell line was eventually found to have a defect in complex I of the mitochondrial electron transport chain, making it unable to reduce NADH in the mitochondria. The elevated levels of NADH in turn inhibited the Krebs cycle enzyme  $\alpha$ -ketoglutarate dehydrogenase by a simple feedback mechanism [18]. The mutation was clearly established to be a nuclear mutation, and a complementing gene was mapped on the X chromosome of mammals [19].

A complete understanding of the phenotype of this first mutant allowed the Scheffler laboratory to devise a selection scheme for the isolation of additional mutants of Chinese hamster fibroblasts in tissue culture with severe defects in respiration [20]. Complementation tests were carried out by pairwise fusions, and the mutants were sorted into seven complementation groups [21]. Similar mutants were subsequently isolated in two other laboratories [22,23] in the course of their search for Chinese hamster cell mutants in tissue culture that would reproduce the phenotype of human cells from galactosemic patients. It was expected that defects in galactokinase or other enzymes in the Leloir pathway would make cells unable to grow in galactose. Clearly, respiration-deficient mutants of the type described above would resemble galactokinase mutants. Reactions of the Leloir pathway are too slow to sustain the high rate of glycolysis required by such mutants. Reviews summarizing the earlier efforts in the various laboratories have been written by Scheffler [24] and Whitfield [22].

A mutant with a severe defect in mitochondrial protein synthesis [25–27] is potentially very interesting. DNA replication and maintenance are unaffected, in contrast to similar mutants of yeast. RNA transcription and processing are also normal, but the failure to translate the mitochondrial mRNAs makes some of these quite unstable, and their steady-state levels are far below normal. cDNAs for mitochondrial elongation factors (mtEF-Tu and EF-Ts) cannot complement the mutation, and a search is in progress to clone the complementing cDNA from a mammalian library and to identify a perhaps novel factor required for translational initiation in mammalian mitochondria.

The biochemical characterization of the specific defect in many of these mutants has been slow. Cloning mammalian genes by complementation with genomic or cDNA libraries is unpredictable and time-consuming. The mutant cells lacking succinate dehydrogenase activity were shown to have a point mutation in the nuclear gene for the anchor protein  $C_{II-3}$ , yielding a truncated peptide, and resulting in the failure to assemble a functional complex II [28,29]. Mutants in two complementation groups with defects in complex I are complemented by normal hamster or mouse X chromosomes [19]. Recent work in I. Scheffler's laboratory has established that the NDUF A1 gene [30] complements one group of mutants. The NDUF A1 gene encodes a peptide of 70 amino acids not found in the core complex I of bacteria, but apparently essential for complex I assembly in mammalian mitochondria.

The demonstration that mammalian fibroblasts can proliferate in tissue culture in the absence of respiration created a basis for the efforts to obtain mutant cells in tissue culture equivalent to the  $\rho^0$  mutants of yeast. Several groups have been successful in such efforts. The first report described  $\rho^0$  chicken cells [31], and subsequently  $\rho^0$  human tumor cells [32,33],  $\rho^0$  mouse cells and  $\rho^0$  primary human fibroblasts have been successfully isolated. The methodology is based on first defining a medium that makes respiration obsolete, and then to use specific inhibitors of mitochondrial DNA replication to deplete cells of mtDNA over a prolonged period of time. Ethidium bromide at sublethal concentrations was used effectively with human tumor cells, but it was less effective with mouse cell lines. Other inhibitors investigated are dideoxycytidine (ddCTP inhibits  $\gamma$ -DNA polymerase in mitochondria), or bisintercalating anti-tumor drugs such as ditercalinium [34].

The  $\rho^0$  human cell lines have found very interesting applications in the study of mitochondrial mutations isolated from human patients, since they can be repopulated with mitochondria from the same species by fusion with enucleated cytoplasts. Thus, mutant mitochondria can be introduced into  $\rho^0$  cells with a different nuclear genome, and effects of mitochondrial mutations can be clearly separated from potential effects of a specific nuclear background. It should be noted that experiments of this type have also given the clearest indication that mitochondria from one species are not compatible with the nuclear genome from another species [35,36]. A more detailed description of such experiments and their usefulness will be presented in a later section.

While yeast *rho*<sup>-</sup> mutants are generated spontaneously at a high frequency, mammalian cells in culture appear to have a stable mitochondrial genome over many generations, and even a mutant cell line with no mitochondrial protein synthesis [25–27] has maintained an intact mtDNA. If there are mutations or deletions, they are present

at a very low frequency. In the early 1970s, when mammalian cells were beginning to be regarded as microorganisms from which useful and informative mutants could be isolated, a challenging question was whether mitochondrial mutations could also be isolated. Few people thought about mtDNA, but those who did were beginning to become aware that there were thousands of copies per cell. A priori, one would therefore predict that a single mutation on one mtDNA molecule in the entire population would not lead to a phenotype, and a phenotype could arise only if there was a mechanism for segregation. It was quite a surprise when the Eisenstadt laboratory announced in 1974 that chloramphenicol resistance in a mouse cell line A9 was cytoplasmically inherited [37]. The target for chloramphenicol were mitochondrial ribosomes, and there was an expectation from experience with bacteria and yeast that one or more mutations in the large rRNA could lead to antibiotic resistance. A year earlier the same laboratory had described chloramphenicol-resistant HeLa cells, but the proof of cytoplasmic inheritance required enucleated cells (by a technique which had just been introduced), and the fusion of such cytoplasts containing the mutated mitochondria with nucleated cells containing wild-type mitochondria. The resulting cells have been referred to as "cybrids." Curiously, the first paper reporting cytoplasmic inheritance concluded that "CAP resistance . . . may be encoded in mtDNA, or possibly in one of the other types of cytoplasmic DNAs reported in mammalian cells . . . (spcDNA, microsome-associated DNA, informational or I-DNA and membrane associated cmdDNA)." Of course the latter types of DNA have now been lost in history, since they most likely were simply contaminants derived from nuclear DNA.

The method of obtaining such mutants is worth recounting. Cells were treated for about a day with ethidium bromide, followed by incubation with 50 µg/ml of chloramphenicol. At 2.5 months later, colonies appeared, which could be cloned and propagated in the presence of the drug. Clearly, at the later stages the cells had a mixed population of mtDNAs with the molecules containing the mutation in the majority. The cells were heteroplasmic. It is necessary to postulate that during the prolonged exposure to the antibiotic the cells initially did not proliferate, and most of the cells in fact died. By a mechanism that is still quite obscure, in some cells a buildup of mutated mtDNA relative to wild-type mtDNA must have occurred. As the proportion increased, mitochondrial protein synthesis could proceed, and eventually the cells gained the level of energy metabolism necessary to sustain cell divisions. With hindsight one might also wonder why such cells did not become *rho*<sup>0</sup> cells, but one must presume that the media chosen would not have supported such cells (glucose level, no uridine or pyruvate present).

The original discoveries were followed by a series of interesting and informative studies on intra- and interspecific hybrids and cybrids [36]. It was shown that the mitochondrial and nuclear genome must be from the same organism to be compatible and to yield viable cells. For example, when CAP-resistant mouse mitochondria were transferred into Chinese hamster cells, the cybrids had a very limited life-span. When interspecies hybrids were made and selected directly in chloramphenicol, the chromosomes of the parent with the resistant mitochondria were retained. In some examples this represents a reversal of the usual loss of human chromosomes from a rodent-human hybrid cell line. By making intraspecies hybrids, it was possible to

make cells that were deliberately heteroplasmic and had a roughly known proportion of wild-type and mutant mtDNAs. Continued culture under selective or nonselective conditions allowed an assessment of the rates of segregation of these genomes from each other under selective pressure or in its absence.

Other antibiotics were used successfully in other laboratories to obtain resistant cell lines in which drug resistance was cytoplasmically inherited [38–41]. Oligomycin (or rutamycin) resistance could arise from a mitochondrial mutation in the “oligomycin-sensitivity-conferring protein” of complex V (ATP synthase), and antimycin resistance resulted from a mutation in the cytochrome b gene encoded by mtDNA. In all cases the cytoplasmic inheritance had to be carefully documented, since other, nuclear alterations (affecting uptake of the drug, for example) could also give rise to such phenotypes. Later, the mutation responsible for oligomycin resistance in Chinese hamster ovary cell mutants was shown to be in the ATPase 6 gene on the mitochondrial DNA [42], and the formation of an altered form of this subunit was also demonstrated by immunological methods [43].

In conclusion, mutations on mammalian mtDNA were first definitively demonstrated in tissue culture cells. The astonishment and continued puzzlement arises not from the existence of such mutations, but about the mechanisms that operate on a population of approximately 1000 mitochondrial genomes per cell to segregate and accumulate a sufficiently large fraction of mutant mtDNAs for a phenotype to be expressed. The extreme selective pressure exerted during the culture with the drugs is responsible for the ultimate selection, but the shift in the mtDNA populations takes weeks or months, while cell division is arrested or extremely slow. This issue will become relevant again in the discussion of human mitochondrial diseases and similar or related mechanisms occurring *in vivo*.

## **7.2 MOLECULAR GENETICS OF HUMAN MITOCHONDRIAL DISEASES**

### **7.2.1 Introduction**

Until about 1988 human patients with myopathies, neuropathies, or encephalomyopathies had attracted attention mainly of neurologists, pathologists, and histologists. Abnormal staining of muscle biopsies viewed in the light microscope had been detected (“ragged red fibers”), and later electron-microscopic investigations had indicated that such abnormalities were associated with morphologically abnormal mitochondria. The term mitochondrial encephalomyopathies (MEM) described a combination of symptoms and morphological characteristics with significant variability and severity, which made a genetic analysis complicated and difficult to interpret. Was it a complex genetic trait? Nevertheless, as examples accumulated and reliable pedigree studies became possible with larger samples, there were indications that MEM may follow a pattern of maternal inheritance. The maternal inheritance of human mtDNA had been firmly established in 1980 [44], after related studies had reached the same conclusion in horse-donkey hybrids [45], in the rat [46,47], in the



white-footed mouse, *Peromyscus polionotus* [48], and in frogs, *Xenopus laevis* [49], and *Drosophila melanogaster* [50]. The ideas converged dramatically in 1988 with two publications describing mutations in mtDNA being linked to “mitochondrial diseases.” In one report, Wallace and coworkers [51] described a point mutation in the ND4 gene (complex I) in several patients with Leber’s hereditary optical neuropathy (LHON). The other, by Holt and colleagues [52], described deletions in mtDNA in patients with mitochondrial myopathies. It has been difficult to keep up with the field ever since. A flood of publications from these and other laboratories rapidly established mtDNA mutations as the genetic basis for a variety of clinical syndromes, in the process defining concepts and generalizations, but also raising new questions of fundamental importance for understanding mitochondrial inheritance and the phenotypic expression of mitochondrial mutations.

The idea of uniparental transmission of mtDNA has recently been challenged in several publications, for species in which intact sperm is found in the egg cytoplasm after fertilization. Backcrosses between *Mus musculus* and *Mus spretus* (an interspecific cross) had yielded offspring in which a very small proportion of paternal mtDNA (0.01–0.1%) could be detected by sensitive PCR techniques [53]. This prompted a reexamination for the much more common intraspecific crosses between mammals, from which Kaneda and colleagues [54] concluded that in intraspecific crosses (*Mus musculus*) the paternal mtDNA is eliminated at the late pronucleus stage. Such a mechanism requires a species-specific recognition system by which egg cytoplasm identifies and destroys sperm mitochondria. Paternal transmission in rare circumstances may be irrelevant for the present discussion, but it will become a potential issue again in the discussion of mitochondria and human evolution [55].

### 7.2.2 Maternal vs Sporadic Inheritance

As one of the pioneers in these investigations pointed out [56], it is perhaps “ironic that the first mtDNA defects identified, large deletions, are not usually inherited,” that is, the observation of maternal inheritance of MEM, though a clue for other disorders, was not relevant in the first studies reported by Holt and colleagues [52]. Patients with MEM had muscle biopsies, and a substantial fraction of such patients (9/25) exhibited heteroplasmy in muscle: up to 7 kb of mtDNA was deleted in 20–70% of the population of mtDNAs. A detailed description of the size of the deletions and the region of the mitochondrial genome affected is given in Section 7.2.3. This heteroplasmy was found originally only in muscle, not in cells obtained from blood. The defects were not maternally inherited. The generation of deletions and the accumulation of deleted mtDNAs in specific tissues may have been a postzygotic event.

As more patients with mtDNA deletions and rearrangements were identified, a fairly complex genetic picture emerged [56,57]. Only one family was found in which the same deletion was transmitted over three generations. In another family, a mother and daughter with similar symptoms had different deletions. The deletions found in several patients could not be detected in their mothers. The child of one patient did not have any deletions detectable by Southern blotting. It is important to recognize that Southern blots may be quite insensitive in the detection of a small fraction of

deleted mtDNA molecules. PCR-based methods are very suitable, if primers are chosen that flank a relatively large deletion. Efficient amplification occurs only when the deletion is present, since in the normal mtDNA the primers are too far apart for standard PCR conditions. In general, this group of mtDNA deletions and the associated symptoms are classified as spontaneous because there is no apparent mendelian pattern of inheritance and there is also no predictable maternal transmission over several generations.

The most likely explanation for the observed distributions of mtDNAs in a pedigree is that the deletions occurred either during oogenesis, leading to the formation of the occasional oocyte with a subpopulation of mutated mtDNAs, or very early during embryogenesis. If the deletion occurred before fertilization, one would expect a widespread distribution of deleted mtDNAs in different tissues. On the other hand, such a distribution could be strongly influenced by unequal segregation of wild-type and mutant mtDNAs during embryogenesis, or by a selection against cells with a large proportion of mutated mtDNAs and hence partially defective mitochondria. It is conceivable that mtDNAs with substantial deletions are replicated more rapidly and thus have a selective advantage over normal mtDNAs. Such an explanation may account for several observations:

1. Depending on the syndrome and its severity, high proportions of deleted mtDNA were found frequently in the muscle and brain (after autopsy, where possible), while other tissues (e.g., liver or blood) had a lower proportion, sometimes only detectable by PCR.
2. The fraction of deleted mtDNAs increases with age in muscle and possibly the brain. In a situation where the total number of muscle fibers does not change appreciably after birth, mitochondrial DNA replication must be dissociated from cell division, selection against defective cells ceases, and shorter mtDNA molecules gain a replicative advantage. Direct tests of such a hypothesis are becoming possible through the use of  $\rho^0$  human cell lines which can be repopulated with mitochondria from enucleated cells with a known proportion of deleted mtDNAs. In one such experiment the fraction of deleted mtDNAs increased from 40–50% to about 80% in a period of 10 weeks.
3. There is a wide range of age of onset of neurological symptoms between birth and the age of 60. The factor determining the age of onset could be the percentage of mutated mtDNAs present in certain tissues at birth, or the rate at which mutated mtDNAs accumulate relative to normal mtDNAs during the period after birth.

A most impressive example of a mtDNA mutation accumulating with time in muscle has recently been reported by Weber and associates [58]. A patient, healthy and athletically active as a teenager, experienced the first symptoms at the age of 34 (joint pains and muscle weakness), and she was subsequently observed and examined over a period of 12 years, during which the symptoms progressively worsened to the point where she is dependent on a wheelchair, and suffers from dysphagia to solids and liq-

uids. Ragged red fibers and COX (complex IV)-negative fibers were observed to increase from 2% and 4.5%, respectively, to 18% and 68%, respectively, over the 12-year period. Finally, mtDNA sequencing established a single-point mutation in the tRNA<sup>Leu(CUN)</sup> gene at position 12,320, affecting the TYC loop at a conserved site. It is difficult to argue that mtDNA with a point mutation has a replicative advantage. The mutation is found in heteroplasmic cells, and restricted to skeletal muscle, the tissue that is clinically and biochemically affected. Within muscle, it was highest in the COX-deficient fibers. In the muscle biopsy specimens that had been collected between 1983 and 1995 the fraction of mutant mitochondrial genomes increased from 0.7 to 0.9. It was absent in fibroblasts and white blood cells. Speculation is that the mutation occurred postzygotically, and may have been restricted to specific cell lineages including myoblasts. However, the significant expansion of the mutation in skeletal muscle is dramatically demonstrated here for a postmitotic tissue.

The patient has daughters over the age of 20, and examining them might be reassuring to confirm that it is indeed a somatic mutation in the mother and hence absent in the offspring, but they have opted not to be tested at this time. This is not an atypical situation encountered in genetic counseling.

The same female patient provided biopsy material to support another very intriguing observation made recently in a number of laboratories [59]. In patients with myopathies caused by mt mutations, even when the mutated mtDNA was present in large excess, progenitor satellite cells contain no, or only very low, levels of mutated mt genomes. Such satellite cells can form regenerated muscle following induction of necrosis. Clark and colleagues [59] were successful in using a local anesthetic, bupivacaine hydrochloride, to induce a localized necrosis, and 3 weeks later the same region of muscle showed clear muscle regeneration, but now a very significant portion of fibers was COX-positive, and in such fibers the level of mutated mtDNA was very low or undetectable. The stimulation of muscle regeneration therefore offers the prospect of reversing severe biochemical and physiological defects in such patients, as an alternative to gene therapy. For obvious reasons, the obstacles to gene therapy with mitochondrial DNA are even greater than those with nuclear genes.

In a pioneering experiment mitochondrial DNA in normal human oocytes has been investigated by Chen and his collaborators [60]. Using sensitive PCR-based techniques these investigators were able to detect rearranged mtDNAs in single oocytes. Among ~100,000 normal mtDNAs, less than 0.1% had deletions, duplications or other structural changes responsible for abnormal PCR products. For most practical reasons one would consider this homoplasmy. But can such a minority population of mtDNAs become the templates from which the embryo is equipped with mutated mitochondrial genomes, possibly differentially in different tissues? A further discussion of this subject is resumed below. The consideration of mtDNA in oocytes has led to an interesting additional speculation. mtDNA deletions have been found to accumulate in an age-related fashion in tissues with slowly or nondividing cells. Oocytes in humans are also arrested (at meiosis I) for a prolonged period of time (years), and it is conceivable that mtDNA deletions might arise during this time. The sample size may be too small at this stage, but one could ask whether the incidence or risk of mitochondrial diseases due to mtDNA deletions could increase with advancing maternal age.

### 7.2.3 Mapping mtDNA Deletions/Rearrangements

A significant effort has been expended to date to map the mtDNA deletions precisely and to define the deletion junctions at high resolution by sequencing. The mapping of the deletions has been pursued with several goals in mind. First, it is desirable to establish precisely which genes or gene functions are affected and to relate such deletions to measurements of residual activities of the mitochondrial electron transport chain. Second, one can ask whether the deletions are entirely random, or whether there are common regions that are most frequently deleted. Finally, if there is a “common deletion,” do the deletion junctions provide any evidence for a likely mechanism by which such deletions could be generated?

The results are provocative, but not straightforward to interpret. Not surprisingly, few deletions have been found so far that delete either one of the origins of replication on either strand, nor have transcriptional promoters been found to be missing. These are the important *cis*-acting elements required for replication, and their loss would also lead quickly to the loss of the mutated mtDNA. Exceptions can be found in the case of a maternally inherited mtDNA deletion (10.4 kb) [62], and in four patients in which the heavy-strand promoter and the adjacent binding site for the transcription factor mtTFA have been deleted [56,63]. Initiation of DNA replication requires transcription from the light-strand promoter, and its continued presence makes the propagation of the deleted mtDNA possible. The two promoters are close together, and deletions starting within the interval are rare. As predicted, and in confirmation of earlier conclusions (see Chapter 4), transcripts dependent on the light-strand promoter were abundant in these muscle fibers, but transcripts from the other strand were missing, regardless of whether the genes were deleted or not [63].

The junctions and flanking regions of mtDNA deletions have been investigated in more than 150 patients (as of 1993; [56]) after amplification by PCR and sequencing. Significantly, in about half the patients there is a “common deletion” of 4.9 kb mapping in the interval between bp 8000 and bp 13,600. More precisely, the sequence up to 8482 is generally retained, and the sequence following 13,460 is also present. Further inspection of the human mtDNA sequence reveals a 13 bp sequence (8470–8482) at one breakpoint region which is directly repeated at the other breakpoint region (13,447–13,459). Loop formation and recombination between the direct repeats is one mechanism suggested by these observations. One plausible mechanism for the formation of such molecules starts with mtDNA dimers that are formed during replication, followed by a recombination event generating the deletion. This model is strengthened by additional data showing that direct repeats of 3–13 bp are found flanking the deleted sequences in 80% to 90% of all the patients examined [56]. Although the model is attractive, it should be considered in light of the knowledge that recombination is generally thought to be absent or very infrequent in mammalian mitochondria, as evidenced also by the lack of active repair systems of the type familiar to us from the nucleus [64]. Therefore, recombination events giving rise to deleted mtDNAs are rare, and, fortunately, patients suffering the consequences are also relatively rare. Another interesting question is whether the postulated recombination activity is restricted to or particularly active at specific stages in the life cycle of an

individual, for example, during rapid mitotic divisions preceding oogenesis, or during the rapid early zygotic divisions in the embryo. Such a restricted period of activity might help explain the origin and distribution of these deletions in families and in different tissues of an affected individual. An alternative to the recombination mechanism for generating deletions is slippage and mispairing during replication of the mtDNA. The issue remains unsettled at this time.

With the mitochondrial genome so packed with genes, every major deletion clearly includes one or more genes, and at this time it is straightforward to predict the consequences at the biochemical level. It should be emphasized that patients with the diagnostic symptoms have been found in which mtDNA deletions were not detectable. Obviously, very small deletions or even single nucleotide changes can cause serious problems. Such single nucleotide changes are discussed below for the cases where maternal inheritance is observed, but clearly, small changes can also occur spontaneously by replication errors, and so far in the discussion the focus has been on spontaneous errors observed in individual patients, and rarely in multiple offspring from the same mother.

A description of the pathologies and symptoms and their relationship to mitochondrial functions is presented later. It need not be stressed that individuals would not be alive if at least some of their mitochondrial genomes were not intact and capable of sustaining the biogenesis of the complexes for oxidative phosphorylation. The generalization can be made that heteroplasmy is the rule in patients with mtDNA deletions.

#### **7.2.4 Maternal Inheritance of mtDNA Point Mutations**

It has been mentioned before that numerous families with incidents of mitochondrial encephalomyopathies show maternal inheritance, a strong hint that an inheritable mitochondrial mutation is involved. The first report with strong experimental support for this genetic basis of the transmission of a disease appeared also in 1988 by Wallace and colleagues [51]. The group at Emory University sequenced most of the mtDNA of a patient with Leber's hereditary optic neuropathy (LHON) and compared it with the published nucleotide sequence for human mtDNA. It is important to point out here, that a total of 25 "mutations" were found, that is, nucleotide differences between the Cambridge sequence and the sequence from an African-American patient with LHON. Which is the deleterious mutation and which represents a harmless single nucleotide polymorphism (SNP or "snip")? An answer to this question continues to be crucial for all correlations of mitochondrial point mutations with a particular disease (symptom), since it is not yet possible to recreate the same mutation by recombinant DNA technology to verify that it alone is responsible for the defect. In 1988 the rationale for identifying the true mutations causing the disease was as follows: Two of the nucleotide changes were in the rRNA genes and thought to be of little consequence; 15 of the changes were base changes, which would not have caused any change in the amino acid sequence of the encoded peptides; no changes were found in any of the tRNA genes in this patient (however, see below), which narrowed the choice to eight potential sites. The extreme variability in metazoan mtDNA sequences, between

closely related species and even within a species, has been noted (see Chapters 4 and 8). Phylogenies can be constructed providing very valuable information on evolution, and, because of the speed of the mitochondrial clock, a time scale of tens of thousands of years can be resolved (see Chapter 8). Investigators had only begun to become aware of the variability among human populations, but sufficient sequence data were available in 1988 to suggest that five of the changes were likely to be of no consequence, since they were also found in other humans not suffering the neuropathy (LHON). Two additional nucleotide changes were found in various ethnic groups and/or in other members of the Georgia pedigree unaffected by LHON. This left the change at nucleotide 11,778 as the likely candidate mutation. A codon change replaces an arginine by a histidine in subunit 4 (ND4 gene) of complex I. The homologous protein has an invariant arginine at that site in species ranging from fungi to fruit flies to humans. Luckily, the nucleotide change also eliminated an SfaNI restriction site found in normal mtDNA, and it became possible to check this site quickly in several other patients of diverse ethnic origin who were afflicted with LHON. All patients with LHON in the limited sample investigated in 1988 had this mutation.

A later analysis employed a parsimony computer program (PAUP) to construct a number of plausible phylogenetic trees for European, African, African-American, and Asian populations, based on nucleotide changes in mtDNA [65]. Details are discussed in a more general context of human evolution in Chapter 8, but here it is relevant that Singh and colleagues demonstrated that the SfaNI mutation at position 11,778 appears to be quite recent, has occurred twice, and in each case was associated with LHON. It was unlikely, therefore, that the mutation at 11,778 was a silent or neutral mutation.

In the intervening years a number of other syndromes with maternal inheritance have been investigated at the molecular level and found to result from single nucleotide changes in mtDNA. Point mutations were found in structural mitochondrial genes as well as in tRNA genes (Table 7.1).

**TABLE 7.1 Some Specific Syndromes Associated With mtDNA Deletions or Point Mutations**

| Clinical Description | Inheritance | RRF | Defect in mtDNA                         |
|----------------------|-------------|-----|-----------------------------------------|
| KSS                  | Sporadic    | +   | Single deletion or insertion            |
| Incomplete KSS       | Sporadic    | +   | Single deletion                         |
| Pearson syndrome     | Sporadic    | +   | Single deletion or insertion            |
| LHON                 | Maternal    | —   | Point mutation in ND4, ND1, CYTB, COI   |
| NARP                 | Maternal    | —   | Point mutation in ATPase 6              |
| MELAS                | Maternal    | +   | Point mutation tRNA <sup>Leu(UUR)</sup> |
| MERRF                | Maternal    | +   | Point mutation tRNA <sup>Lys</sup>      |
| MIMyCa               | Maternal    | +   | Point mutation tRNA <sup>Leu(UUR)</sup> |

RRF, ragged red fibers in muscle; KSS, Kearns-Sayre syndrome; LHON, Leber's hereditary optic neuropathy; NARP, neuropathy, ataxia, retinitis pigmentosa; MELAS, mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes; MERRF, myoclonus epilepsy and ragged red fibers; MIMyCa, maternally inherited disorder with adult-onset myopathy and cardiomyopathy.

Before describing LHON and other defined clinical syndromes with maternal inheritance in more detail from a biochemical and physiological standpoint, some important distinctions between single-point mutations and larger deletions must be emphasized. Individuals with point mutations in their mtDNA are generally heteroplasmic, but frequently they are homoplasmic, that is, all mtDNAs examined contain the mutation. This astonishing observation immediately raises two questions, one less challenging, the other difficult to answer at this time. How can such individuals survive? How does homoplasmy come about?

The first question can be answered theoretically and experimentally simply by showing that the point mutation leads to a "leaky" phenotype, a protein or a tRNA that is still partially functional. Another possibility is that the mutation in a tRNA affects the rate or efficiency of maturation of individual mRNAs from the polycistronic transcript and hence mRNA levels with no effect on tRNA function. While this answer may satisfy temporarily, it immediately generates more questions, especially in the case of homoplasmy. First, one of the outstanding and most challenging questions in the whole field is to explain why only certain tissues are affected; in the most extreme case, why, for example, do patients with LHON suffer the central vision loss (although other symptoms have also been observed). Second, the optic atrophy (similar to other syndromes discussed here) is delayed in onset, which is variable among affected individuals in a pedigree. Third, the severity of the vision loss is also variable. Finally, pedigrees harboring the more severe mutations in a homoplasmic state can include family members who are not affected at all.

The distinction between severe and mild mutations is in part based on an assessment of the amino acid substitution and the effect it might have on the residual activity of the peptide. A Leu to Pro change observed in one pedigree with homoplasmy is expressed in 80% of the individuals of the family, presumably because of a drastic alteration in the conformation of the peptide. Where amino acids are conserved in evolution, functional or spatial restraints are indicated, and changes at such positions are expected to be more serious. Changes found in nonconserved regions of the peptide are difficult to evaluate in the absence of a complete structure of the complex.

In more formal genetic terminology one can speak of the expressivity of the mutation in individuals within affected families and the penetrance of the mutation in the general population. Penetrance is defined as the probability of detecting a particular combination of genes (mutations) when they are present (in the population or in a pedigree). This may depend on the criteria and means of observation (measurement). In other words, the degree of penetrance of a gene is a measure of the fraction of individuals possessing the mutation and showing manifestations of the mutation. Expressivity refers to the degree to which a particular phenotype due to a particular mutation manifests its presence. "Expressivity refers to the type of manifestation of a gene that is penetrant" [65a]. Both parameters are most likely dependent on other genes, and in the present discussion the expression of mitochondrial mutations will depend on a combination of nuclear genes.

While the possibility of the existence of homoplasmy in viable individuals can be explained on the basis of a leaky mutation, the development of homoplasmy for a mutation in a rare pedigree in the human population is more difficult to explain. In al-

most all normal individuals ~99.9% of mtDNAs are identical with no harmful mutations. In its simplest form the problem can be stated as follows: A rare point mutation occurs in a mtDNA in germline cells of a female. At the time of origin the mutated mtDNA is one among hundreds if not thousands of normal mtDNAs. How many generations does it take to accumulate a significant fraction of mutated mtDNAs (heteroplasmy)? How and how quickly is homoplasmy achieved?

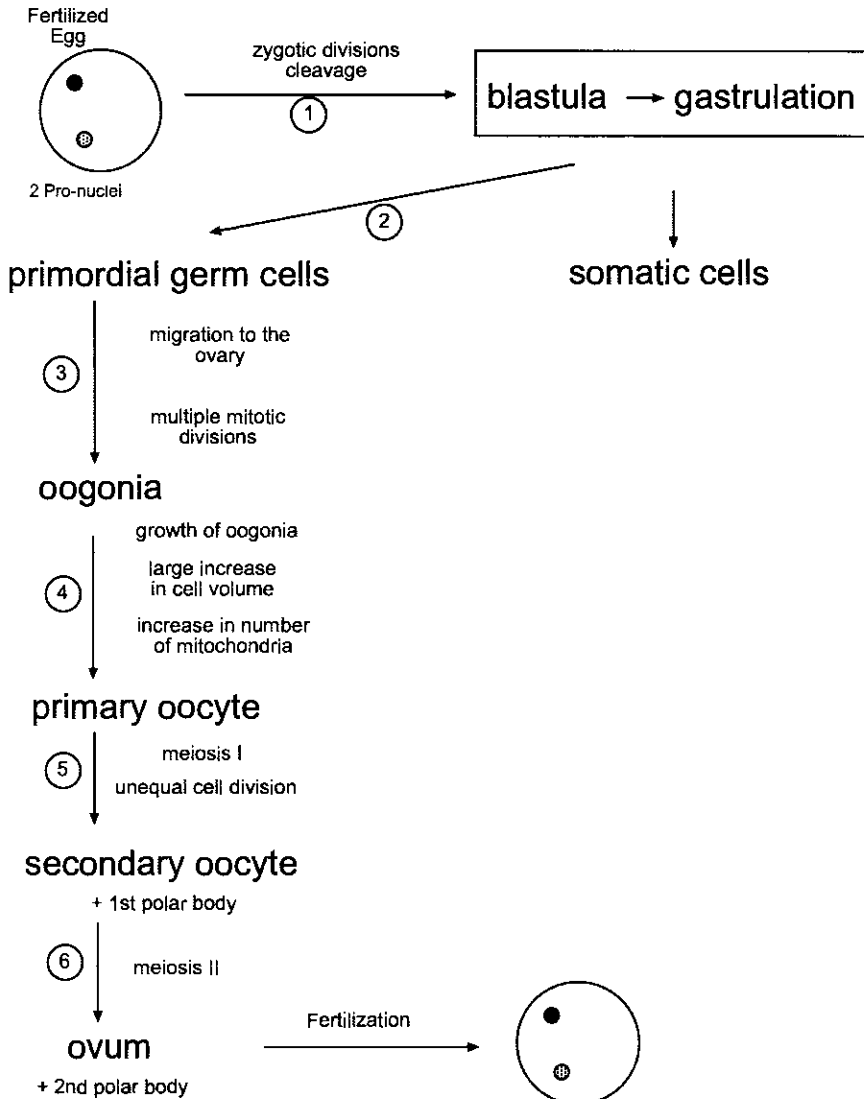
There are indications that a significant change in the mitochondrial genotype can occur within a few or even a single generation in mammals. Hauswirth, Laipis, and colleagues [66,67] have taken advantage of a common polymorphism in a herd of Holstein cows to demonstrate that a switch between alleles differing by point mutations was frequently almost complete from one generation to the next. In other words, homoplasmy with respect to a specific base defining the alleles was achieved in the change and transmission from mother to offspring.

### 7.2.5 Mitochondria and Oogenesis

To escape from this conundrum, investigators have proposed the idea of a “bottleneck”: a very small number or even a single mtDNA serve as templates for replication to populate the oocytes from which the mtDNA is transmitted to the offspring [68,69]. A recent critical review has challenged the use of the term “bottleneck” because it can be confusing in its meaning and has been used inconsistently by different authors. Instead, the term “sampling and amplification” has been suggested by Lightowlers and colleagues [70]. They also point out that while either random drift or a complex sampling process can readily explain a rapid segregation of mitochondrial genomes, the real puzzle is the stable heteroplasmy observed in some pedigrees. A female with a relatively small fraction of mutated mtDNAs can have offspring with a significantly higher proportion of mutated mtDNAs, if the mutated DNA is preferentially amplified. But unless there is bias in the sampling, in the majority of her offspring the heteroplasmy should be reduced.

At what stage in gametogenesis or embryogenesis is the sampling and amplification mechanism operational? A priori several stages could be considered (Figure 7.1). One should be aware that the human egg has an estimated 100,000 mtDNAs [60], and that as much as a third of the total DNA of an oocyte is mtDNA. There is certainly no “bottleneck” at this level, in the sense that only a small number of mitochondria are transmitted to or by the oocyte. After fertilization and before implantation a number of rapid zygotic (mitotic) divisions occur (Figure 7.1, step 1), apparently without any further mtDNA replication, in which the pool of oocyte mitochondria is distributed between the daughter cells and the number of mtDNAs per cell is reduced to the normal range of 1000 or less [71]. It is conceivable that the partitioning of the embryonic cells between the inner cell mass and the extraembryonic tissues is accompanied by an unequal partitioning of mtDNAs which could be responsible for the segregation of mtDNA genotypes. In this hypothesis segregation is a postzygotic event. At a relatively early stage in development a primordial germ cell lineage is set aside (Figure 7.1, step 2) to migrate to the ovary, and within the ovary a subpopulation of diploid cells (oogonia) is formed by repeated mitotic divisions (Figure 7.1, step 3). The oogo-





**Figure 7.1** Mitochondrial DNA amplification and relationship to oogenesis. The fertilized egg contains an estimated 100,000 mtDNAs. These may be distributed during the early zygotic divisions (*step 1*) to yield cells with considerably smaller populations of mtDNA. Early in development germ cells are differentiated from somatic cells (*step 2*). A very limited number of mtDNAs could be replicated during *step 3* (expansion of the precursor pool of oogonia by mitotic divisions), or in *step 4* when oogonia increase in size and prepare for meiosis.

nia increase significantly in size, and a multiplication in the number of mitochondria could accompany this growth (Figure 7.1, step 4). Meiosis and maturation leads to one ovum (and polar bodies) from one oogonium. (It should be noted that in mammals meiosis I is arrested, possibly for years, and completed only at periodic intervals when eggs mature. Furthermore, meiosis II is subsequently arrested and completed only if fertilization by a mature sperm takes place.) If the estimated 100-fold amplification of mtDNA molecules during the maturation of the primary oocyte from an oogonial precursor cell involved only a small subpopulation of mtDNA templates ("sampling and amplification"), shifts in the distribution of "alleles" could be achieved quite rapidly. To restate the two a priori hypotheses, sampling and amplification could occur postzygotically, or in the process of formation of an oocyte from an oogonial cell. A third possibility has emerged from preliminary recent experiments that suggest the sampling may occur during a series of mitotic divisions leading to a large population of oogonia (Figure 7.1, step 3).

Several experimental systems have been developed in recent years to achieve "cracks in the bottleneck" [68]. Advances in the manipulation of mouse embryos have been instrumental in creating the kind of experimental conditions allowing a direct observation of the segregation of mtDNAs in heteroplasmic mice. Jenuth and colleagues [72] produced mouse embryos by the fusion of cytoplasts containing mitochondria of one genotype with one-cell embryos of a strain with another mitochondrial genotype. For simplicity, the mtDNA genotypes will be referred to as NZB and BALB, respectively. The cytoplasts were obtained from one-cell embryos by using a glass pipette to draw up and pinch off cytoplasm surrounded by plasma membrane. These were injected under the zona pellucida of recipient one-cell embryos and electrofused. Transplantation into pseudopregnant females produced female and male animals whose mtDNA could be analyzed from tail biopsies. Five founder females were produced with 3.1–7.1% of the donor mtDNA genotype. When a female with 5% donor (NZB) mtDNA was crossed to a BALB male, 28 offspring were analyzed and found to contain, on the average, 6.7% NZB mtDNA. However, in individual offspring the proportion of donor (NZB) mtDNA varied from 0% to 29.6%. A rigorous statistical analysis of 191  $F_1$  offspring from all five crosses, and 176 offspring from several more backcrosses with  $F_1$  females indicated a large variance of genotype frequencies. It was concluded that the results supported the idea that germline segregation is a stochastic process capable of producing significant fluctuations from an original distribution of genotypes.

The analysis was extended to a comparison of the distribution of mtDNA genotypes in mature oocytes of several founder females, and in the  $F_1$  offspring of the same founder females. No statistically significant differences were found in the ranges of donor mtDNA fractions or in the coefficients of variation, leading to the conclusion that the stochastic segregation postulated does not take place during post-fertilization development. It must take place at an earlier step, that is, during the development of the oocyte itself. Thus, frequencies and variances in the frequencies of mtDNA genotypes in primary oocytes from immature heteroplasmic mice (2–3 days after birth) were compared to primary oocytes from mature, adult mice. Surprisingly, the difference in the coefficients of variation was insignificant. In an experimental

tour de force the oocytes from one ovary were removed for analysis on postnatal day 2, while the other ovary from the same mouse was transplanted and allowed to mature in the ovarian capsule of an immunodeficient mouse. A subsequent comparison of genotype frequencies confirmed the earlier conclusions. These observations failed to support either of the two a priori hypotheses and pushed the time frame for the stochastic segregation of mitochondrial genomes to an even earlier developmental stage. Before such hypotheses are dismissed completely, it should be realized that the experiments are very demanding and difficult, and more experiments are required before a firm conclusion can be reached.

In mice approximately 50 primordial germ cells can be identified in 7.5-day mouse embryos. When several (3–12) such primordial germ cells from a given embryo were analyzed, the coefficient of variation was very small, suggesting that a given proportion of donor mtDNAs received in the zygote was maintained during the earliest stages up to the establishment of the germ cell lineage. These primordial germ cells, when properly located on the genital ridge, divide and give rise to oogonial cells, from which approximately 25,000 primary oocytes are derived in a series of synchronous divisions. Based on these experimental results Jenuth and colleagues [72] propose that segregation of mtDNA variants must occur during the mitotic cell divisions that precede meiosis in the ovary.

At this point one can only speculate about details of the process [69,72]. Several aspects of mtDNA replication must be considered (see Chapter 4). Unlike the case of the nuclear genome, mtDNA replication is relaxed, that is, in a given population of mtDNAs some may replicate multiple times during the cell cycle, while others do not replicate at all. The necessary nuclear factors may be present in a very limited amount. A possible candidate for a regulatory role is the transcriptional activator mtTFA, since it controls the synthesis of the RNA primer required for replication from the  $O_H$  origin (see Chapter 4). This still leaves the question: how many mtDNA molecules are selected for replication, and is there any kind of selection for a subpopulation? There is strong evidence that localization within a cell can influence mtDNA replication, proximity to the nucleus being a determining parameter [73]. Mathematical modeling using population genetic theory, and assuming that segregation is the result of genetic drift, one can calculate the effective number of segregating units,  $N$ , from the observed variance in the  $n$ th generation if the initial distribution of genomes is known. With an estimated 15 cell divisions required to produce the total population of primary oocytes in the mouse ovary, Jenuth and colleagues [72] calculated that  $N$  is in the range of 200, and they point out that this is also close to the estimated number of mtDNAs based on experimental measurements of the total number of mitochondria.

The numbers for the mouse will undoubtedly be refined, and additional experiments will be done to confirm whether genetic drift during the mitotic expansion of a pool of mammalian oogonia to form primary oocytes can account completely for the observed mtDNA segregation. The comparison of the data obtained for the mouse [72] with those obtained for Holstein cows [67] already suggests some significant difference (fivefold) in the estimate of the effective number of segregating units [72]. It was pointed out that the polymorphism in the cows was in the D-loop sequence, and

it was questioned whether the results for the cows could be influenced by a high mutation rate or genetic instability in the specific region. Since the D loop also contains important cis-acting control elements, one could imagine that the selection of mtDNA molecules for replication could be biased.

In the case of humans, the distinction between variants with point mutations and variants with large deletions is an important one. The fact that changes are occurring postfertilization is clearly indicated by the differences in heteroplasmy observed between muscle tissue and blood cells in patients with mtDNA deletions. Homoplasmic cells with deleted mtDNA would not be expected to be viable. On the other hand, if replication factors are limiting, and only a subset of mtDNAs are replicated in a cell cycle, the smaller size may not constitute a replicative advantage because completion of replication is not a rate-limiting step.

## 7.2.6 Clinical Aspects of mtDNA Mutations

### 7.2.6.1 *mtDNA Deletions: Kearns Sayre Syndrome and Pearson Syndrome*

It need not be stressed that homoplasmic null mutations would be lethal. Two distinct mechanisms can lead to partial deficiency. In heteroplasmic cells the most severe mutation, a complete deletion of one or more genes, can be partially compensated by the presence of normal mitochondrial genomes. In homoplasmic cells, a mutation must be less severe, for example, by causing a missense substitution in a protein, which reduces its activity. In either case, one expects the tissue to suffer from an insufficient production of ATP by oxidative phosphorylation, and one may now ask what is it that distinguishes the various "mitochondrial diseases" from each other. A priori, one would expect the severity of the disease to depend on the degree to which a particular function is compromised. In the same vein, one would expect the deficiency to be most deleterious in tissues such as the brain and muscle, which are energetically the most demanding. In practice, the situation is much more complicated in ways that continue to challenge our understanding of the basic phenomena.

Large-scale rearrangements or deletions of mtDNA are associated with two clinical conditions which share overlapping symptoms. The first, recognized by Kearns and Sayre in 1958 [74], includes among the symptoms retinitis pigmentosa, external ophthalmoplegia, and a complete heart block. Morphological alterations in the brain were also noted at autopsy, and these have since been confirmed as the rule rather than an exception or artifact. The disease is characterized by a childhood onset, progressive ophthalmoparesis, and pigmentary retinopathy, increased propensity of complete heart block (which could lead to sudden death), neurological symptoms including incoordination, mental retardation, and episodic coma. Growth retardation and hearing loss are common, and ragged red fibers are found in almost all affected patients. An unbalanced and insufficient energy metabolism is indicated by elevated lactate and pyruvate levels in plasma. Named after its original discoverers, the syndrome is now referred to as the Kearns-Sayre syndrome, or KSS. As explained earlier, the occurrence of KSS is sporadic not familial, tissues are heteroplasmic with respect to wild-type and rearranged mtDNA, and both sexes are affected at about equal frequencies.

A second syndrome named after its discoverer is Pearson syndrome [75]. The affected infants had pancytopenia and altered pancreatic exocrine function, and it was fatal in some cases, but others survived into adulthood and developed clinical features of KSS. When molecular genetic analyses became possible, lesions in mtDNA were found very similar to those found in KSS. How is this syndrome survivable, only to be converted into the second severe condition? Speculation centers around cell population dynamics in the bone marrow, where cells with the greater proportion of mutated mtDNAs have a disadvantage during rapid cell divisions and may be eliminated, that is, in this tissue there may be a selection for cells with the highest proportion of normal mtDNAs. At the same time, in tissues with nondividing cells like muscle or the brain there may be a selection at the level of mtDNA replication which favors the deleted mtDNAs, and ultimately an oxidative phosphorylation deficiency is developed concomitant with encephalomyopathy. Patients diagnosed in infancy with Pearson syndrome do not have ragged red fibers, whereas muscle biopsies showing an abundance of ragged red fibers also show deleted mtDNAs in excess.

Patients with Pearson syndrome and those KSS patients with the most severe symptoms have detectable heteroplasmy not only in muscle, but also in fibroblasts and leukocytes, tissues easily available from biopsies. At autopsy, brain and liver tissue (and other tissues) become available, with the brain showing pronounced heteroplasmy or an abundance of mutated mtDNAs, while liver may have a smaller proportion. On the one hand, the ubiquitous presence of mutated mtDNA is an indication that the mutation was present in the oocyte before fertilization, but the variability in the ratio of normal to mutated DNA in different tissues is indicative of perhaps two selection mechanisms based on the fitness of individual cells in rapidly dividing or renewing tissue (blood, liver), and the replicative advantage of mutated mtDNA molecules in nondividing tissue. As a result, these distributions may vary between patients and most significantly, may shift with age. Delayed onset and variable expressivity may be explained quite satisfactorily in general terms, but more definite predictions at the time of the first diagnosis may not be possible. Even a determination of the proportion of mutated DNA in a muscle biopsy (which muscle?) may not have any predictive value, since there is no correlation between this fraction and the clinical severity.

It is commonly assumed that there are several mtDNAs per mitochondrion, and that intramitochondrial complementation occurs. Continuous fission and fusion of mitochondria would be expected to contribute to a mixing of the two genomes. However, such a picture, derived from observations in tissue culture, may be misleading in tissues such as muscle, where the highly structured cytoplasm and the cytoskeleton (sarcomeres) may prevent extensive diffusion and mixing of mitochondria.

The severity and the precise combination of symptoms may also be dependent on the nature of the rearrangement and the specific segment of mtDNA missing in heteroplasmic cells. Nevertheless, for reasons explained above, patients with identical deletions may exhibit different clinical abnormalities. Almost any deletion of some length will cause the loss of several genes, including structural genes encoding essential subunits for complexes I, II, and IV, alone or in combination, and almost always the loss of at least one tRNA gene. Thus, very specific predictable biochemical

activities will be compromised, but the loss of one or more tRNA genes will also diminish the capacity for mitochondrial protein synthesis, and hence the level of all complexes of the electron transport chain may be affected. A more detailed discussion of several specific cases can be found in the review of Harding [56]. Superimposed on the effects of the specific deletions are mechanisms of posttranscriptional regulation such as mRNA stability and the control of steady-state levels of individual mRNAs, and translational control mechanisms (initiation?), which may influence the half-life of an mRNA. If such mechanisms are tissue-specific, a simple consideration of heteroplasmy and gene dose is definitely not sufficient to predict the full scope of clinical symptoms and their severity.

#### **7.2.6.2 Familial Mitochondrial DNA Depletion**

A rare disorder with an inevitably fatal outcome in early childhood has been studied in several families. Healthy parents have produced children suffering a severe depletion of mtDNA in specific tissues, most frequently liver or muscle. First recognized by Moraes and coworkers [76] as a novel genetic abnormality, the defect appears to reside in a nuclear gene, since no mitochondrial mutations could be detected. Furthermore, mitochondria from such a patient could be introduced into  $\rho^0$  cells and repopulate such cells with mtDNA [77]. Thus, the unaffected parents were believed to be heterozygous for a recessive mutant allele, a conclusion strengthened by the finding of two affected sons and an affected daughter in a consanguineous marriage [78]. However, a more complex inheritance could be indicated by the discovery of one father with two affected children from two different mothers. The restriction of the loss of mtDNA to specific tissues is still much of a puzzle, especially since variable tissue involvement has been reported in a single family.

Because affected families are rare and usually small, linkage studies are difficult. One approach has been to select candidate genes and carry out haplotype analysis with closely linked satellite markers. Among the candidates, the genes for the mitochondrial transcription factor (mtTFA), the nuclear respiratory factor 1 (NRF-1), the mitochondrial single-stranded DNA binding protein (mtSSBP) and endonuclease G were examined, but so far only mtTFA remains a candidate, if inheritance is indeed recessive [78].

#### **7.2.6.3 Point Mutations**

Table 7.1 lists five distinct clinical conditions resulting from point mutations in mtDNA that are maternally inherited: LHON, NARP, MELAS, MERRF, and MIMyCa. A genetically interesting and challenging feature of these diseases is the frequent finding of homoplasmy (see discussion in Section 7.2), and a perhaps oversimplified aspect of each mutation is that it is "leaky," which in biochemical terms implies that some function is retained. A detailed consideration of each condition and patients having the mutation leave many other questions still unanswered.

**7.2.6.3.1 Leber's Hereditary Optical Neuropathy** Leber's hereditary optic neuropathy (LHON) has as the defining feature the sudden onset of bilateral central vision loss. The condition was first described by Leber in 1871. Onset is usually

delayed until young adulthood (20–24 years of age), but may occur as early as age 5. Although bilateral, vision may be affected first in one eye, followed by involvement of the other eye weeks or months later. Individuals are generally healthy, although the mutation is found in all tissues. Detailed histological and anatomical studies have established the specific degeneration of the ganglion cell layer and the optic nerve. Although vision loss is permanent in most cases, a number of patients with LHON have shown improvement, sometimes relatively soon after the diagnosis, sometimes after several years. Disedema and subtle alterations in retinal vessels are part of the diagnosis, but curiously, the clinical condition is not followed rapidly by the appearance of other neurologic abnormalities, although patients (and family members) have exhibited some or all of the following: hyperreflexia, Babinski signs, incoordination, peripheral neuropathy, and cardiac conduction abnormalities. How this highly restricted pattern of degeneration of the optic nerve is initiated is still a matter of debate, which has recently been summarized by Howell [79]. A detailed discussion requires the description of the microanatomy of the optic nerve head, the region around the lamina cribosa and the possibility of a “chokepoint” in the optic nerve where axoplasmic transport of mitochondria may be impaired. The resulting swelling may accentuate ischemia, and ultimately neurodegeneration. Patients do not have ragged red fibers in muscle, and lactate levels in blood and cerebrospinal fluid are normal.

Members of some families, predominantly females, have been reported to be at risk for developing symptoms resembling multiple sclerosis (MS). The risk of LHON is inherited from the mother, typical of mitochondrial diseases, and the mother may or may not have been affected. The primary mutation (see below) in most patients with LHON is homoplasmic, but patients with heteroplasmy have also been found. There is incomplete penetrance even in the homoplasmic population, and the current view is that the primary mutation predisposes toward LHON, but secondary genetic and environmental etiological factors contribute to the development of the disease [80]. 85% or more of the patients are male [80]. Historically, this fact was thought to indicate X-linkage, with the appearance in a larger than expected fraction of females requiring an explanation. Today the maternal inheritance is firmly established, challenging us to explain what genetic factor(s) contribute to the biased expression of this phenotype. An X-linked susceptibility locus has been proposed to play a role. However, while the issue has not been definitively resolved, recent studies have failed to obtain evidence for such a locus on the X chromosome. On the other hand, an X-linked gene encoding a peptide of complex I has been identified [19,30].

In their review Brown and Wallace [81] have described 16 point mutations associated with LHON, in subunits ND1, ND2, ND4, and ND6 of complex I, in CYT b, and in the CO1 subunit of complex IV. Brown and Wallace also express the view that mtDNA LHON mutations “represent a continuum of severity of OXPHOS defects with each imparting a proportional increased risk for LHON.” The issue is controversial, and Howell [80] emphasizes that the vast majority of families who suffer from LHON have one of three primary pathogenic mutations in mtDNA at nucleotides 3460 (ND1 gene), 11,778 (ND4 gene), and 14,484 (ND6 gene). The most abundant mutation in the ND4 gene at nucleotide 11,778 converts a highly conserved arginine to a histidine. Other, less common mitochondrial mutations may have a secondary eti-

ological role, or they may appear in rare “nonclassical” LHON families suffering from optic neuropathy and additional neurological abnormalities. Another perspective is that mild “LHON mutations” may act synergistically. This is deduced from the finding that at least four “LHON mutations” by themselves do not cause loss of vision, but, in combination with other “LHON mutations,” the phenotype is expressed in almost 100% of individuals carrying the two mutations. The probability of such double mutants would at first seem extremely small, until it is realized that some of these mutations are present in a substantial fraction of the general population (0.1% to 10%, probably variable in different ethnic groups) [82]. Additional data on population genetics and history will be required to establish whether other rare mutations are mildly pathogenic, or secondary, or of no consequence at all. It will be important to resolve whether LHON is caused exclusively by mutations affecting complex I.

While there is agreement that the primary mutations in the ND1, ND4, and ND6 genes represent the major risk factor in LHON, there is more controversy about the actual effect of these mutations on the rate of electron transport and oxidative phosphorylation. It would seem straightforward to look at the specific activity of complex I (the NADH-CoQ oxidoreductase) in mitochondria from patients with LHON and normal control subjects. Nevertheless, results in different laboratories have been contradictory, depending on what was measured [summarized in Reference 80]. One type of measurement involves oxygen consumption with NADH-linked substrates, and respiration with succinate used as a control and for normalizations. Care must be taken to measure complex I activity by demonstrating the sensitivity to rotenone. Experiments can be performed with white blood cells and platelets from patients, or after construction of cybrid lines containing mitochondria from patients with LHON and nuclei from  $p^0$  lines. Another approach is to measure the pyruvate:lactate ratio as an indicator of the NADH/NAD balance in fibroblasts. The method has pitfalls discussed by Howell [80].

Why does a reduced activity of complex I in homoplasmic patients cause LHON, while a reduction in electron transport and OXPHOS capacity in a patient with heteroplasmy and mtDNA deletions cause KSS? In homoplasmic patients who have LHON there is no mechanism for a further shift toward a worse condition in individual cells or tissues. By contrast, in patients with KSS, sampling of available tissue may indicate 50% heteroplasmy, for example, but this is an average, and individual cells in the brain or myotubes in muscle may be more severely compromised, causing clinical symptoms. It is clear that much remains to be learned about the distinction between localized defects in individual complexes of the electron transport chain and an overall reduction in several or all complexes due to a mtDNA deletion. The metabolic consequences are almost certainly more complex than a simple reduction in ATP supply.

**7.2.6.3.2 Neuropathy, Ataxia, Retinitis Pigmentosa** If there is a “bottleneck” in oxidative phosphorylation it can easily be imagined in complex V, as already indicated by the well-known phenomenon of coupling in mitochondria and the dependence of electron transport on the ADP concentration. A more rigorous statement is that ATP turnover (synthesis and consumption) has the highest control coefficient of



all the reactions of OXPHOS, and changes in this rate would be expected to have the largest effect on the overall rate of oxidative phosphorylation (see Chapter 5). One may, therefore, be able to rationalize that mutations in ATP synthase (complex V) subunits are rare, and in the one reported case the symptoms are relatively broad. A mutation in subunit 6 (nucleotide 8993; Leu → Arg) leads to neuropathy, ataxia, and retinitis pigmentosa (NARP). Since the structure of complex V is beginning to become resolved at high resolution (see Chapter 5) the amino acid substitution can be related to the observed inhibition in ATP synthesis: an additional positive charge is positioned in the proton channel. In fact, the inhibition appears to be severe, and patients with NARP are heteroplasmic, with the severity of the symptoms related to the fraction of mutated genomes. At one end of the spectrum are mild neurological signs such as migraine headaches and retinitis pigmentosa, and at the other end one observes necrotizing encephalopathy or Leigh syndrome [82–84]. A pedigree has been described in which the mother was mildly affected (87% mutant mtDNA), but all four offspring had >90% mutant DNA and were severely affected; two daughters died of Leigh syndrome at an early age.

**7.2.6.3.3 MELAS, MERRF, and MIMyCa** Point mutations responsible for mitochondrial encephalomyopathy with lactic acidosis and strokelike episodes (MELAS), myoclonus epilepsy and ragged red fibers (MERRF), and maternally inherited disorder with adult-onset myopathy and cardiomyopathy (MIMyCa) are found in specific tRNA genes. The MERRF mutation (tRNA<sup>Lys</sup>) is heteroplasmic. Although the symptoms vary from mild (hearing loss, mitochondrial myopathy, electrophysiological changes) to severe (uncontrolled myoclonic jerking, dementia, cardiomyopathy, nephropathy, neurosensory hearing loss, altered VER and EEG), there is no strict correlation with the percentage of mutant mtDNAs. In the view of Wallace and his colleagues [85], age strongly influences the expression of the mutation. This group has become the strongest advocate of the hypothesis that an accumulation of mitochondrial mutations in somatic cells with age causes a general decline in OXPHOS capacity, possibly at different rates in different tissues. For each tissue there is a critical threshold below which the energy needs of the cells can no longer be met. In an individual starting out with a lower capacity due to a tRNA<sup>Lys</sup> mutation, the drop in capacity with age will reach the threshold sooner, and symptoms start to appear. The nervous system and muscle with the highest minimal requirements are predictably the first tissue to be affected [82]. Based on the observations in pedigrees, an individual with >90% of mutant tRNA<sup>Lys</sup> could nevertheless be normal at a young age, but would deteriorate as he or she aged. This hypothesis has to some extent prompted research and is now supported by data showing an increase in mtDNA mutations with normal aging in various tissues (see Section 7.3).

## 7.2.7 Conclusion

Who can ever experience a migraine headache or muscle fatigue without at least a thought of mitochondria, even though the cause may not always be a mutation. A little over a decade ago physicians may have had a distant memory of mitochondria from the

basic biochemistry course in their first year in medical school. Today histochemical and molecular tests of mitochondrial dysfunction have become almost routine in the analysis of a broad spectrum of neuropathies and myopathies. Both nuclear and mitochondrial mutations can contribute to defective mitochondria. Severe nuclear mutations in homozygous individuals would almost certainly be lethal. For example, only a handful of individuals with defects in complex II have been reported [86,87]. If heterozygotes have no increased fitness or selective advantage (as may be the case in sickle cell anemia and possibly in the case of cystic fibrosis), and homozygotes are not viable, the allele frequency in the population may remain small. Nevertheless, the alert medical community in the developed countries has increasingly simpler and cheaper means of screening millions of patients for potential mutations, and the number of "mitochondrial mutations" will undoubtedly increase significantly in years to come.

### 7.3 MITOCHONDRIAL DNA AND AGING

The most important inventions in evolution are sex and death. (F. Jacob)

#### 7.3.1 Introduction

The achievements of the last decade have revitalized research on mitochondria, not only because new insights gained into the genetic basis of mitochondrial function and dysfunction, but also because of the realization of their vital role in maintaining a healthy individual. It is, with hindsight, perhaps not too surprising that mitochondria as the "powerhouse" of the cell would play a critical role in the health (function and survival) of cells. Turnover of all cellular components is a constant activity, consuming as much as 50% of the total energy supply, and in the broad sense turnover is clearly related to "maintenance and repair." When the energy supply becomes limiting, the infrastructure slowly deteriorates, and more severe consequences may be triggered by catastrophic failures at localized sites. This view, particularly as related to the process of aging, is undoubtedly very simplistic. Nevertheless, the concept that aging and mitochondrial dysfunction may be related has had a powerful appeal to a number of investigators in the past decade [88–92]. This chapter attempts to review some of the hard facts and follow up on some of the more promising speculations. A discussion of the many aspects of senescence is clearly beyond the scope of this treatise. Familiar characteristics associated with aging are a decline of muscle power and often but not always diminished mental capacities. From the well-established cause-and-effect relationship between clinically significant encephalomyopathies and mitochondrial DNA mutations, it is tempting to look for a similar relationship between normal aging and a reduced capacity for respiration and oxidative phosphorylation.

Some clear distinctions should be made at the outset. Attempting to define a single cause for normal aging may be presumptuous. However, it is probably not too far-fetched to claim that a diminution of mitochondrial functions may either be central to the process, or be a strong contributor influencing the timing and the severity of the overall deterioration. The challenge is to set up a plausible chain of events and to es-

tablish a cause-and-effect-relationship. A credible scenario gaining acceptance is based on the postulate of a slow and stochastic buildup of damage caused by reactive oxygen species (ROS), which are a normal by-product of respiration. Superoxide, hydrogen peroxide, and hydroxyl radicals are constantly being formed. It is estimated that up to 4% of the oxygen consumed in mitochondria is converted to ROS. Their potential harmfulness can be judged from the coevolution of a set of enzymatic defense mechanisms such as superoxide dismutase, catalase, and peroxidase, enzymes that destroy these reactive species before damage can be done. Additional substances (e.g., vitamin E) can act as free-radical scavengers, and they have been proposed for megavitamin therapies. Although the largest proportion of oxygen utilized by a cell is by the mitochondrial electron transport chain, cytochrome P450, cytosolic oxidases, and the oxidations in the peroxisome generate reactive oxygen species outside the mitochondria, and the protective functions are found in multiple compartments.

The reactive oxygen species can damage DNA, proteins, or lipids. Thus, it is at least conceivable that the primary cumulative damage is to mitochondrial lipids (e.g., cardiolipin), altering membrane fluidity and ultimately causing defects in electron transport and respiration; as a result, the generation of reactive oxygen species may be accelerated. Eventually, defense mechanisms and repair systems are overwhelmed and damage to mtDNA becomes permanent. Alternatively, one could consider mtDNA to be the primary target of superoxide or hydroxyl radicals, and as more and more mtDNAs sustain mutations in critical coding regions, complexes in the electron transport chain become less efficient or inactive, leading to a decline in mitochondrial function. In evaluating these alternatives (among still others), attention must be paid to the relative rates of turnover of the major mitochondrial constituents. Generalizations applying to all differentiated cells and tissues are likely to be inappropriate.

It is informative to document and measure the changes in mtDNA populations that accompany the aging process, and in some cases such data will provide an important baseline for comparisons with the corresponding data from abnormal clinical samples. There are also several well-known and prevalent neurodegenerative diseases that are most frequently occurring with advancing age, Parkinson's disease, Huntington's disease, and Alzheimer's disease among them. Patients of all types have been extensively examined for abnormalities in electron transport and oxidative phosphorylation, as it seemed more promising with these disorders to look for a primary cause at the level of mitochondria.

### **7.3.2 Accumulation of mtDNA Damage and Normal Aging**

The idea that the accumulation of mtDNA damage might be part of, or even responsible for normal senescence is a relatively novel concept. If it was expressed before, it would have been pure speculation, since the technical means for testing it were not available until the late 1980s. The first prerequisite was the determination of the human mtDNA sequence. The second challenge was to find, characterize, and quantitate abnormal mtDNA molecules present at low abundance in a population of 1000 to 3000 molecules per cell. Restriction mapping can detect only large rearrangements

or deletions in heteroplasmic populations when the abnormal molecules are a homogeneous subpopulation and present as a significant fraction (10%?). A heterogeneous mutant population with many different point mutations, each present at the <1% level is not so easily detectable.

As in so many other areas, the polymerase chain reaction has had an enormous impact on the analysis of mitochondrial genomes. Taking a cue from the observations in patients with myopathies, it became possible to focus the initial attention on relatively large deletions, and specifically the ~5-kb “common deletion” between the repeats at 8470 and 13,447 frequently found in patients with Kearns-Sayre syndrome (see Section 7.2.6.1). By the appropriate choice of oligonucleotide primers such deletions can be detected when present in just a few mtDNA molecules (0.1–1%). Following the initial observations in the laboratory of Linnane [93; reviewed in References 90 and 91], several laboratories have investigated the incidence of age-related deletions in a variety of human tissues including brain, heart, kidney, liver, assorted muscle, and blood [90,91,94–98].

Several generalizations can be made:

1. As suspected, there is an age-related increase in the frequency of mtDNA deletions.
2. Deletions are detected readily in nondividing tissue such as brain and skeletal muscle, while in cells with a relatively short half-life (e.g., blood cells) few if any mtDNA deletions have been detected. There must be a selection against an accumulation of damage in rapidly dividing stem-cell populations, or alternatively, metabolically deficient cells are eliminated even more rapidly than by normal turnover. The underlying assumption, that mtDNA turns over even in nondividing cells, is based on an observation made as long ago as 1969 [99], but there is a dearth of information on how fast this turnover occurs in such cells.
3. Different deletions may accumulate in different tissues of the same individual. Such an observation lends strong support to the hypothesis that the observed mutations are the result of stochastic processes in somatic tissue.
4. The range and nature of the deletions vary from individual to individual; however, as discussed in a different context, the presence of short repeats in the mt genome may be responsible for the mechanism generating the deletions by recombination or “slip-replication” [100], and therefore the deletions are not entirely random.
5. The tissues of an individual accumulate deletions at different rates, that is, they do not “age” uniformly.
6. Even within a particular tissue, such as skeletal muscle or cardiac muscle, a tissue mosaic pattern has been observed by histochemical staining reactions. For example, staining for cytochrome oxidase activity in cardiomyocytes in a large collection of specimens has shown rather uniform staining in samples from young subjects, but in samples from older individuals the incidence of weakly stained or even unstained myocytes was roughly related to the age of the indi-

vidual [101]. While provocative and consistent with present thinking, it should be stressed that the variable levels of cytochrome oxidase activity have not been related directly to mtDNA mutations.

Sequencing of PCR-amplified segments (clones) of mtDNA from a given tissue is a tedious and time-consuming process, and hence the accumulation of point mutations in aging tissues is less well documented, and the rate of generation of such mutations is still more difficult to determine.

Apart from errors resulting from illegitimate recombination or replication, mtDNA damage is very likely also caused by chemical reactions, and it has been suggested by numerous investigators that reactive oxygen radicals generated as a by-product of respiration may make a very substantial contribution to mtDNA damage. Free radicals are known to be potent mutagens that can induce base changes and modifications. It has been suggested that three factors make mtDNA particularly vulnerable: 1) The mtDNA is localized close to the inner membrane, where free radicals are produced; 2) mtDNA is not extensively condensed and protected by histones; 3) the DNA repair activity is limited (see Chapter 4). The resulting point mutations (after replication) may be difficult to find (see above), but the reaction with free radicals (superoxide and peroxide) may also leave modified bases in the DNA which can be detected by sensitive biochemical means. Several reviews on the subject have been written by Ames and colleagues [102–104]. A representative example is 8-hydroxyguanosine, which has been shown to accumulate in mtDNA of the human diaphragm in individuals of advanced age [105]. Approximately 0.5% of the guanine residues had been converted to 8-hydroxyguanine in an 85-year-old individual. In a related study, the fraction of derivatized guanines was 0.87% in the brain of a 90-year-old person. Covalent DNA alterations termed I-compounds have also been described by Gupta and coworkers [106] in the liver of aged rats.

Covalent modifications of DNA in the nucleus are readily detected by DNA repair systems, but there is agreement that some types of DNA repair cannot be found in mammalian mitochondria. A continuous exposure to free radicals is therefore expected to be damaging over the long run, and the extent of such damage would be expected to be more severe in actively respiring tissue compared to less active tissue. This idea has been extended in an investigation of the effects of physical activity and age on mitochondrial function [107], and specifically in the muscle from elderly athletes [108]. Brierley and colleagues make the important comment that the observed decline in respiratory chain function in human skeletal muscle from older individuals is significantly greater than the observed level of actual mutations. In a carefully selected and matched group of individuals there was no correlation between oxidative metabolism and chronological age. Instead, the results suggested that physical activity contributes greatly to the maintenance of OXPHOS capacity, or in other words, exercise may counteract and mask the effect of accumulated mtDNA damage.

In order to assess the contribution of mutated mtDNA molecules in the population present in “old cells,” that is, cells derived from older individuals, several informative experiments have been performed with the help of  $\rho^0$  mutant mammalian cells [109,110]. The  $\rho^0$  cells were derived from 143B.TK<sup>-</sup> cells, an immortal human os-

teoblastoma cell line. Such  $\rho^0$  cells do not harbor any mtDNA of their own, and they can be repopulated with mitochondria under investigation by fusion with an enucleated donor cell. Such an experiment has the additional advantage that it removes the nucleus from the old donor cell, and hence eliminates the influence of nuclear genes. The results of an elaborate series of fusions and analyses have recently been published by Laderman and colleagues [110]. Donor cytoplasts were from individuals ranging from 20 weeks of age (fetal) to more than 100 years. Almost 200 clones were investigated with respect to growth rate and respiration rate. Every clone was also analyzed to determine the mtDNA levels per cell, since parallel experiments had shown that the "transformation" of  $\rho^0$  cells with mtDNA is quite variable and slow, and oxygen consumption measurements had to be compared with, if not normalized to, the mtDNA content of the cells. Thus, several clones derived from the same mtDNA donor were also compared, and considerable variation in respiratory capacity as well as mtDNA levels were observed. The final evaluation of the data required careful statistical analysis, because at least two distinct age-related phenomena were observed. First, the number of mtDNA molecules in the cybrids was lower, when the enucleated donor cells were from older individuals. Second, the same subgroup of cybrids also exhibited a lower respiration rate. Notably, however, there was no correlation between oxygen consumption and mtDNA content of individual clones. Laderman and colleagues speculated about an increase in age-dependent mtDNA mutations which could affect the functional properties of the complexes, the translational machinery of the mitochondria (rRNAs or tRNAs), or even promoter sequences controlling transcriptional efficiency. Such damage would not necessarily affect DNA replication. On the other hand, mutations in a region crucial for DNA replication could have affected the efficiency of transforming the  $\rho^0$  cells with mtDNA.

These results clearly support the hypothesis of a functional deterioration of mtDNA with age, since presumably all mitochondrial proteins encoded by nuclear genes and lipids from the old enucleated cell will have been replaced at the time of the analysis. One may also wonder why not all transformants derived from old cells were partially respiration-deficient, and the answer is most likely that aging is accompanied by stochastic mtDNA mutations, with some cells being much less affected than others. Finally, it should be noted that any such *in vitro* experiments require the establishment of clonal cell populations of sufficient size to be able to perform the analyses, and hence the cells must grow, albeit at more or less reduced rates. Selections and segregation were considered by Laderman and colleagues by following the long-term behavior of some of the clones, which, initially, had been analyzed 8 to 18 weeks after fusion. Additional changes were observed in some cases.

The cybrids described above are genetically related to the human osteoblastoma tumor cells. Do such tumor cell lines accumulate mtDNA mutations after extensive subculture *in vitro*, and are such mutations continuously subject to counter selection? A comparison of the mtDNA sequence of a 143B.TK<sup>-</sup> cell with the mtDNA sequence in the original tumor and in normal somatic tissue would perhaps be interesting. Alternatively, mtDNA sequences from HeLa cell strains kept for decades in different laboratories would also be worthy of investigation to learn why mtDNA mutations do not seem to accumulate significantly in rapidly proliferating cells.

### 7.3.3 Parkinson's Disease

Much remains to be learned about Parkinson's disease, although it is a relatively common disorder, affecting approximately 1 in 40 individuals over a lifetime. Clinical symptoms include bradykinesia, rigidity, and tremor. The most specific and characteristic pathological condition is the loss of dopaminergic cells in the substantia nigra of the brain. It can be stated at the outset that there is no evidence for maternal inheritance in PD, and the analysis of mtDNA in brain has not revealed any major abnormalities such as deletions or rearrangements, or consistent point mutations that can be associated with PD (see below).

One of the most intriguing observations hinting at a mitochondrial involvement in PD was made serendipitously. An illegally produced "designer drug," MPTP (1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine), an injectable pethidine analogue, was found to be neurotoxic and to induce parkinsonism in users [111,112]. Further experimental support for this analysis came from the induction of very similar symptoms in primates. It was discovered that the drug specifically destroyed dopaminergic cells in the substantia nigra. MPTP is converted to the active metabolite 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>) by monoamine oxidase in glial cells, and MPP<sup>+</sup> is transported into dopaminergic neurons by the dopamine reuptake pathway. It can be concentrated in mitochondria at millimolar concentrations by a process dependent on a membrane potential, and there it acts as a specific inhibitor of complex I. A more detailed discussion of MPP<sup>+</sup> and its inhibition of complex I can be found in Chapter 5. For the present discussion it suffices to state that inhibition of complex I activity has been postulated to be responsible for dopaminergic cell death.

What makes drug-induced parkinsonism so relevant is the substantial body of evidence showing that in the substantia nigra of PD patients complex I activity is reduced 30–40% relative to controls, but this reduction is not found in other brain regions [reviewed in Reference 113]. Mitochondrial abnormalities, and specifically reductions in complex I activity, have also been investigated in muscle and platelets of PD patients. The results and their interpretation are still somewhat controversial.

Since 7 of more than 40 polypeptides of complex I are encoded by mtDNA, looking for mitochondrial mutations in PD patients became imperative. A few reports on more detailed mtDNA sequence analyses have appeared, including at least one with an examination of the entire mtDNA sequence of several patients [114]. One was a PD patient, but three others had overlapping symptoms for PD and Alzheimer's disease (AD). The conclusion was that there was no single mtDNA mutation shared by all the patients. In particular, earlier studies at Emory University and by Hutchin and Cortopassi [115] had found an apparent prevalence (7- to 20-fold increase) of a mutation at position 4336 (tRNA<sup>Gln</sup>) in AD or AD + PD patients, compared to race- and age-matched controls, and phylogenetic analysis. Some patients were found to be variants in ND1 or ND5. As the sample size increased, the risk factor for AD associated with the 4336 mutation actually decreased [115]. The most definitive statement that can be made at this time about these mutations is that they may contribute to the pathology of AD and/or PD, but they are not found in all patients and are also found in normal controls.

### 7.3.4 Alzheimer's Disease

AD is an increasingly common form of dementia in our population in which the life expectancy has been raised significantly over the past decades. It is a progressive disease linked to the premature death of cholinergic neurons in the brain. The primary event responsible for this destruction has remained elusive, although it is likely that multiple genetic and environmental factors can either trigger it or influence the age of onset and the rate of progression. In the cases where there is early onset and a familial pattern of inheritance the products of three nuclear genes have been implicated. Autosomal dominant mutations have been mapped on chromosomes 21 (APP), 14 (PS-1), and 19 (ApoE), and the  $\beta$ -amyloid precursor protein (APP) as well as the presenilin 1 and 2 proteins (PS-1, -2) are currently subject to an intense research effort [reviewed in References 116–119]. In the case of the more common, late-onset AD a genetic component has been indicated by some tantalizing observations, but the uncertainty is exemplified by the statement that “the lack of a family history is a negative risk factor for AD.” One possible risk factor identified to date is the prevalence of the apolipoprotein E type 4 allele among affected individuals [reviewed in Reference 117]. The delayed onset and age-related incidence of the disease has made it very tempting to establish a connection to mitochondrial mutations. Indeed, the existence of a maternal relative with the disease is associated with an increased risk of AD, indicative of a link to maternal inheritance and mitochondria. Numerous studies have attempted to solidify the basis for a role of the mitochondrial genotype in AD. The results are intriguing, but their significance is still being hotly debated.

The 4336 mutation (tRNA<sup>Gln</sup>) studied also in connection with Parkinson's disease (see above) occurs in 4.6% of Caucasian patients with neurological disease, but in only 0.3% of age-matched controls [115]. Since the risk of developing AD approaches 10% at over 65 and 50% at age 90 or older, the mitochondrial mutation cannot account for the observed risk, although more comprehensive statistical analyses may show that it pushes the age of onset downward.

Much attention was attracted by a study correlating mutations in two mitochondrial cytochrome oxidase genes with late-onset AD [120]. Several results were striking and even puzzling. A follow-up study in two independent laboratories concluded that the presumed COX mutations were found in a pseudogene of mtDNA transferred to the nucleus [121,122]. In a related but less extensive study the mitochondria from 33 AD patients and 30 age- and sex-matched controls were examined. The activity of four complexes (I–IV) were assayed in mitochondria obtained directly from lymphocytes. None of the patients had respiratory chain enzyme activities below the normal range. Thus, the relationship of cytochrome oxidase activity to AD remains controversial.

A recent review [117] concludes that overproduction and increased aggregation of the amyloid  $\beta$  peptides may be an obligatory early step in the development of the disease, followed by deposition of diffuse plaques, which in turn activate glia and astrocytes to release cytokines and acute-phase proteins. Direct neurotoxicity of the amyloid peptides or the induced “inflammatory” changes lead to numerous metabolic and cytoskeletal alterations, including changes in calcium homeostasis, and injury to



mitochondrial functions. A fundamental question is therefore whether mitochondrial (OXPHOS) dysfunction is a secondary effect or one of the primary causes. A particular mitochondrial mutation may either predispose toward Alzheimer's disease, or it may influence the severity and/or onset of the disease. To decide between these two alternatives remains one of the challenges of the future.

### 7.3.5 Huntington's Disease

Huntington's disease is well characterized at the genetic level as an autosomal dominant disease with 100% penetrance. The responsible gene (IT-15) has been mapped on chromosome 4, and the mutation belongs to the class of trinucleotide repeat polymorphisms (CAG), first identified in fragile X individuals. The resulting abnormal protein, huntingtin, with an expanded polyglutamine tract, must represent a gain in function to explain the dominant phenotype. Its normal function is still not completely clear, and the mechanism of the gain in function (or enhancement of a normal function) is also obscure at this time. It appears to interact with calmodulin in the presence of calcium. Inappropriate apoptosis triggered by abnormal huntingtin has been proposed [123]. Its expression in a wide variety of tissues including brain, testes, liver, heart and skeletal muscle, lung, among others, suggests that it has a very general function outside of the nervous system, but selective neuronal cell death remains to be explained.

A possible relationship to mitochondrial dysfunction is deduced very indirectly from the late onset of the disease, and possibly from the neurodegeneration observed in affected individuals. Measurements of biochemical activities in mitochondria of HD patients have also been suggestive, but here, even more than in PD and AD, one has to approach the problem from the point of view that the mitochondrial deficiencies are clearly secondary. Studies measuring OXPHOS enzymatic activities in selected brain tissue have to be interpreted with care, since the problem of normalization is not a trivial one. If a subpopulation of cells degenerate and disappear, the remaining cells may have normal activities. A more reliable determination is the measurement of an intrinsic property, namely the fraction of mtDNAs in a given tissue with point mutations or deletions. Seeking to ascertain whether the progression of HD is correlated with an accelerated accumulation of somatic mtDNA mutations, and specifically the mtDNA4977 deletion (the "common deletion"), Horton and associates [124] indeed found that HD patients have an order of magnitude higher levels of this deletion in the frontal and temporal lobes of the cerebral cortex compared to age matched controls. There was no statistically meaningful difference in the corresponding comparison for the occipital lobe and the putamen. The latter tissue is atrophied in the most in advanced stages of the disease. The apparent paradox is hypothesized to be explained by the death of the neurons containing the highest levels of mutations. With such a scenario, any analysis must be made at a time when the integrity of mtDNA or the activity of mitochondrial enzymes is already in decline, but before the damage is sufficient to kill the cells.

A biochemical link between mutated huntingtin and energy metabolism is indicated by the following intriguing observations. The polyglutamine tract of huntingtin

has been shown to bind to glyceraldehyde-3-phosphate dehydrogenase, a key enzyme in glycolysis. It is not yet clear whether this interaction is directly responsible for the hypometabolism of glucose in HD brains [125]. In conjunction with decreased activities of complexes II and III in mitochondria of HD brain (caudate) this excess metabolism of glucose causes lactic acidosis and elevated lactate/pyruvate ratios in HD cerebrospinal fluid. Animal models resembling HD have been induced by the complex II inhibitors malonate or 3-nitropropionic acid. In light of the discussion above, it is also noteworthy that elevated lactate levels and decreased phosphocreatine/inorganic phosphate ratios were found in tissue (gastrocnemius, calf muscle) where abnormal huntingtin is expressed without causing an obvious pathology or cell death. Thus it has been proposed that impaired oxidative phosphorylation may predispose neurons to elimination by excitotoxicity [125].

Until the biological activity and function of huntingtin and the gain of function of the mutant protein is understood in more detail, the death of neurons could have many causes. It is worthwhile to be reminded that apoptosis, programmed cell death, is a common and perfectly natural biological and developmental process. Mitochondria or mitochondrial proteins have been implicated in this process. A definition of a primary cause-and-effect relationship continues to be an elusive goal in this field as well.

## 7.4 MITOCHONDRIA AND APOPTOSIS

### 7.4.1 Introduction

Although cell death can occur as the result of a variety of causes, including accidental failure of an incubator, it is “programmed cell death” that has attracted enormous attention in recent years. The program referred to in this context is a genetically controlled pathway and mechanism that can eliminate unwanted cells under three major circumstances: 1) development and homeostasis, 2) a defense mechanism against genetically damaged and hence potentially tumorigenic cells, and 3) natural senescence and aging. The phenomenon is primarily found in multicellular, eukaryotic organisms, although it has been argued that cell death may have evolved in single-celled organisms as a defense strategy [126]. By committing suicide, a cell may eliminate itself as a host for a parasitic invader, and hence protect related, neighboring cells from the spread of the pathogen. In higher organisms programmed cell death or apoptosis is now recognized as an essential aspect of development, and many examples could be cited. Many neurons die within a developing brain, self-reactive T lymphocytes are eliminated during the development of the immune system [127], the morphogenesis of limbs requires cell death as part of the shaping of the final limb such as a hand, and a most detailed description of the cells destined to die has been worked out for all the lineages in a developing worm, *C. elegans*. The immune system can take advantage of and activate an existing program to kill infected cells. In the adult, apoptosis ensures that there is turnover and repair in many tissues without continued growth, and certain other tissues may expand or contract reversibly in response to hormones and cytokines. Another aspect of apoptosis is that this program is inhibited or

inactivated in tumor cells. Hence some genes playing a role in this mechanism were initially identified as oncogenes in tumor cells. Overexpression of bcl-2 in hematopoietic tumor cells, for example, inhibits cell death normally induced in growth factor-deprived B cells and others. Inducing apoptosis specifically in tumor cells has become a major new strategy in the fight against cancer, as opposed to inhibition of tumor cell proliferation [128].

A number of isolated systems were initially studied without an obvious connection, but now the emphasis is frequently on the common mechanisms found in many cells across a broad spectrum of species, and on homologous genes encoding functions associated with apoptosis. For example, treatment of lymphocytes with glucocorticoids has long been known to initiate a suicide program, but the generality of the mechanism was not recognized until later. A major boost to the field also came from the observation that very specific cells in the developing nematode *C. elegans* would die during development, and that specific mutations could identify genes (*ced* = cell death abnormal) required for this process. This observation stimulated the search for homologous genes in other organisms and made the nematode a powerful model system for studies of programmed cell death. A detailed description of the physiological processes in different organisms is beyond the scope of this treatise. It is to be reemphasized that the programmed cell death is orchestrated by genes within the affected cell, and that common genetic pathways may have evolved over a wide phylogenetic range. Nevertheless, since most of this research has been performed with mammalian cells, the emphasis in the following discussion will be on mammalian systems. A most relevant aspect of the phenomenon for the present discussion has been the postulated involvement of mitochondria or mitochondrial functions.

It has become useful to subdivide the process of apoptosis into several phases or stages. The final phase encompasses a number of characteristic changes such as cell shrinkage, chromatin degradation and nuclear fragmentation, and loss of plasma membrane integrity. It appears that these changes are essential for rapid recognition of the dying cell by its neighbors, which phagocytose the remnants and efficiently eliminate any debris which would otherwise cause an inflammatory response by the immune system. As a consequence, "dead" cells from apoptosis are not readily seen in vivo in intact tissue, and the description of this terminal phase rests largely on observations in tissue culture.

The first phase includes the description of the various stimuli or signals which induce apoptosis in different cells. The stimulus may be an external one, received by a cell surface receptor, or an internal one, as the result of the action of a drug, toxin, or radiation damage. Of course, drugs, toxins, and radiation are also ultimately external influences, but the difference between these two mechanisms becomes apparent during the following phase: the signal transduction which leads to changes in metabolic state, induction of various enzymes (proteases, nucleases), and the morphological changes preceding cell death. Needless to say, these two signaling pathways may converge at some level, but at the same time the above description should not give the impression of a simple, universal and invariable pathway operating in all cells under all conditions. Deprivation of growth factors (e.g., interleukin-3 for hematopoietic progenitor cells) induces apoptosis, that is, unoccupied receptors can stimulate apopto-

sis. Failure to stimulate germinal center B cells by antigens causes them to die. Transforming growth factor  $\beta$  and tumor necrosis factor can trigger apoptosis in various cell types. Many more examples could be given [126,129]. It should be clear that these signal transduction pathways include most if not all of the following known reactions, intermediates, and interactions: tyrosine kinases, phosphatases, steroid receptors, inositol phosphates, transcriptional activators and factors, and so on.

There is usually a significant time interval (several hours) between the delivery of the first stimulus and the observation of morphological changes in the targeted cells. It is also during this interval that thresholds or checkpoints are reached beyond which the process becomes irreversible. Many details of events occurring during this effector phase remain elusive and are the subject of a very active research.

#### 7.4.2 The Bcl-2 Protein and the Permeability Transition

As mentioned above, the nematode *C. elegans* continues to be a useful model system, and it was in this model system that the first genes specifically associated with apoptosis were isolated (*ced1*, *ced-2*, *ced-3*, . . . , *ced-10*, etc.). Many of these genes were subsequently shown to be required for the disposal of the dead cell(s), and therefore their detailed description will be omitted here. The *Ced-3* gene, however, was identified to encode a cysteine protease, and six different homologues have since been found in mammalian cells. The first was interleukin-1 $\beta$ -converting enzyme (ICE), and this group of aspartate-specific cysteine proteases is often referred to as ICE<sub>x</sub>, where *x* is a distinguishing subscript. More recently this family of proteases has been referred to as caspases. In a simplified view, apoptosis is triggered by proteolytic cleavage, and there is in fact a cascade of such cleavages, since these ICE enzymes exist as inactive precursors (procaspase) that must be cleaved after a specific aspartate residue before they can be assembled into heterotetramers and active proteases. The other targets of these proteases are still largely unknown, although it was intriguing that one of these proteases (CPP32) can cleave the poly-(ADP ribose) polymerase (PARP), an enzyme involved in DNA repair. However, PARP knockout mice develop normally. The ICE proteases are constitutively expressed in many cells, and their activation is a posttranslational event, although some additional transcriptional activation following the death stimulus may also occur. It is apparent, therefore, that there must be an upstream signal for posttranslational activation which remains to be more clearly defined in mammalian cells. Certain broad-range protease inhibitors can prevent apoptosis in response to common stimuli, but other highly specific and restricted inhibitors may interfere with the signaling pathway initiated from only a limited number or a unique inducer. Apoptosis induction may have "common" as well as "private" pathways.

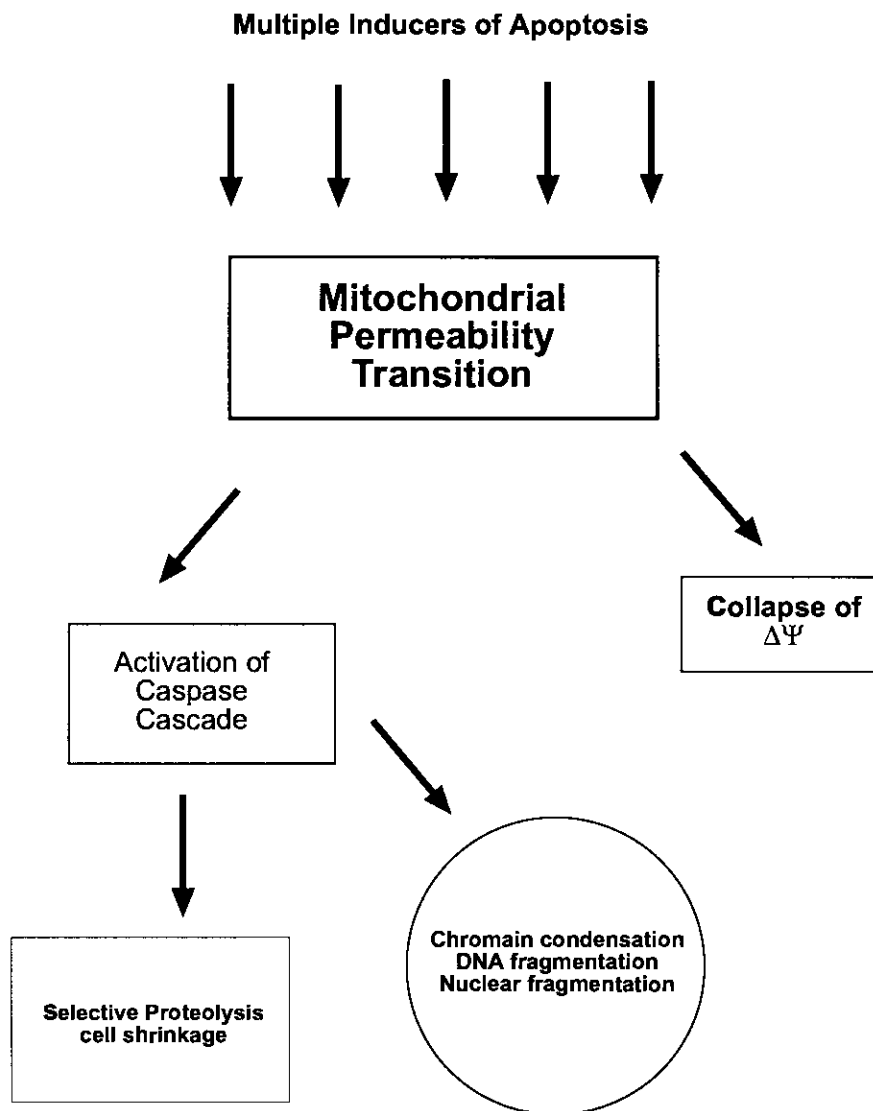
The *ced-9* mutation in nematodes led to the isolation of the corresponding nematode gene and the potential functional identification of the human homologue Bcl-2. The Bcl-2 protein was localized in the outer mitochondrial membrane (although see below), and this finding was one of the first links to mitochondria. The mammalian Bcl-2 gene was initially implicated in tumorigenesis because it was found to be the location of a breakpoint (18q21) in a reciprocal translocation found persistently in

human B-cell tumors [130–133]. Bcl-2 protein became overexpressed under the influence of an immunoglobulin promoter. A whole family of bcl-2-like proteins (Bcl-2, Bcl-x<sub>L</sub>, Mcl-1, Bfl-1, A1,...) were subsequently defined and characterized as inhibitors of apoptosis by transfection studies and overexpression in various cells [133]. Other family members have also been localized in the outer mitochondrial membrane [134]. In addition, factors promoting apoptosis have also been found: Bax, Bcl-x<sub>s</sub>, Bad, Bak, Bik, . . . , etc.

Before discussing Bcl-2 and mitochondria in more detail, some other approaches related to the study of apoptosis and mitochondria must be introduced. Active, healthy mitochondria exhibit a transmembrane potential ( $\Delta\Psi_m$ ) across the inner mitochondrial membrane which is of fundamental importance in the discussion of the mechanism of oxidative phosphorylation (see Chapter 5). A pH and electrochemical gradient across the inner membrane is set up as the result of electron transport through the electron transport chain, with excess positive charge on the outside, that is, in the intermembrane space. The discovery was made some years ago that certain lipophilic dyes such as rhodamine 123, 3,3'-dihexyloxacarbocyanine iodide (DiOC6(3)), 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1), and chloromethyl-X-rosamine (CMXRos), (see Chapter 5 for a more detailed description), can be accumulated in mitochondria and fluoresce. Under controlled conditions fluorescence intensity can be correlated with the magnitude of the membrane potential ( $\Delta\Psi_m$ ), and these dyes have therefore been used as convenient and powerful indicators of mitochondrial  $\Delta\Psi_m$ . When such dyes were applied to cells undergoing apoptosis, a fall in  $\Delta\Psi_m$  was observed as one of the relatively early events in many different cell types, prior to DNA fragmentation. In fact, the collapse of the transmembrane potential was correlated in time with the point of no return, that is, the point at which apoptosis could no longer be reversed by withdrawal of the stimulus [135]. The breakdown of the  $\Delta\Psi_m$  can be shown to involve the so-called mitochondrial "permeability transition" (PT) [136]. A more detailed discussion of the permeability transition is found in Chapter 5. Briefly, it is believed to be the result of the opening of a PT pore, which can be measured electrophysiologically by the opening of a "megachannel," large enough to allow solutes of up to 1500 Da to pass through along a concentration gradient. This PT pore is regulated and has diverse functions as a voltage sensor, thiol sensor, redox sensor, pH sensor, sensor of adenine nucleotide levels, and regulator of calcium homeostasis. Normally only a brief opening or transient assembly of a small number of such pores may be required, but the massive opening of many such pores will collapse the  $\Delta\Psi_m$ , stop oxidative phosphorylation, arrest the import of proteins into mitochondria, and permit leakage of cytochrome c out into the cytosol. Thus, a long-term, massive opening of the PT pores can be expected to do permanent damage to mitochondria, and the PT has been postulated to be a central, rate-limiting event in the apoptotic cascade [134–136]. The damage may be accentuated by a depletion of GSH levels and therefore oxidation of sulfhydryl groups, and by the increased production of reactive oxygen species, doing further damage to nucleic acids, lipids, and proteins.

A recent review [137] with a focus on novel, p53-induced gene products ascribes to them a role in generating a burst of reactive oxygen species "on the mitochondrial

membrane, permitting them to both release the safety catch (Bcl-2) and pull the triggers (Bax, Ced-4, AIF) of the terminal effector pathway of apoptosis." It concludes with the question: "... does the murder take place in the nucleus or in the mitochondrion?" It seems unresolved whether the reactive oxygen species are an obligatory intermediate or a symptom, a weapon used to commit suicide or a signal to trigger the protease cascade (Figure 7.2).



**Figure 7.2** Schematic view of signaling in apoptosis. In this view the mitochondrial permeability transition takes a central position on which multiple early pathways converge. [Reviewed in Reference 132.]

That the collapse of  $\Delta\Psi_m$  is one of the major events in the effector phase of apoptosis is indicated by other observations in various systems when the inducing stimulus is followed by an inhibitory signal: progression towards apoptosis cannot be separated from the breakdown of  $\Delta\Psi_m$ . For example, inhibition of transcription and protein synthesis inhibits the permeability transition and apoptosis of lymphocytes induced by glucocorticoids. An inhibitor of the ICE protease prevents  $\Delta\Psi_m$  collapse and apoptosis, and similarly, a knockout of the p53 tumor suppressor gene, or overexpression of bcl-2 in transfected cells is effective in preventing both the  $\Delta\Psi_m$  breakdown and apoptosis. The relationship between the breakdown of  $\Delta\Psi_m$ , the opening of PT pores, and apoptosis can also be shown by the use of other inhibitors. Cyclosporin, an immunosuppressive drug, has been shown to act on cyclophilin (a peptidyl-prolyl-*cis-trans* isomerase) in the mitochondrial matrix and cause a transient inhibition of the PT. When given to cells which have received a death signal, the  $\Delta\Psi_m$  collapse is prevented. A similar effect can be achieved with bongkreikic acid, an inhibitor of the adenine nucleotide translocator (ANT) in the inner mitochondrial membrane. The latter result can be explained by the participation in the formation of the PT pore complex, but the precise composition and structure of the pore is still unknown (see Chapter 5 for further discussion).

In the same vein, the Bcl-2 protein has been postulated to be associated with the PT pore complex, and thus its inhibitory action on apoptosis could be rationalized. The arguments in favor of the protective action of Bcl-2 occurring through a mitochondrial mechanism have been summarized by Kroemer and colleagues [134]. They rest mainly on its localization in the outer mitochondrial membrane to which it is targeted by a C-terminal signal anchor sequence. Its distribution within the membrane is patchy, and possibly restricted to the contact sites between the outer and inner mitochondrial membranes. It is left open whether these contact sites represent PT pores or transient assemblies of the Tom and Tim machinery for protein import (Chapter 4). Are these two structures distinct? Another curious aspect is that Bcl-2 has also been located in the nuclear membrane and in the endoplasmic reticulum. Such a dispersion seems to violate the dogma about unique addresses specified for each protein by means of signal sequences. It has been stated that the localization of Bcl-2 varies depending on the cell type, but all authors agree on the association with a membrane via the C-terminal 19 hydrophobic amino acid stretch [133]. Wherever it is found, the largest domain of the protein extends into the cytosol, where it appears to be capable of interacting with other proteins, for example, in its formation of a heterodimer with Bax.

It is clear that a truncation of the Bcl-2 protein deleting its C-terminal transmembrane (anchor) sequence makes the protein incapable of suppressing apoptosis induced by several agents. Furthermore, this anchor sequence can be replaced by the corresponding anchor sequence from the yeast outer membrane protein Mas70 (now Tom70), and Bcl-2 targeting and function are restored. A number of studies have shown that overexpressing Bcl-2 in transfected cells can enhance the steady state  $\Delta\Psi_m$ , prevent  $\Delta\Psi_m$  disruption, and affect even the behavior of isolated mitochondria. It prevents not only the collapse of  $\Delta\Psi_m$ , but also the loss of calcium, and other molecules from the mitochondria.

When the three-dimensional structure of the Bcl-2 homologue, Bcl-x<sub>L</sub>, was determined, a similarity to the pore-forming domains of diphtheria toxin and the bacterial colicins became apparent. This prompted a number of recent studies aimed at testing channel formation by Bcl-2 or Bax proteins in liposomes and unilamellar lipid vesicles or in planar lipid bilayers [138,139]. In acidic lipid membranes and at low pH recombinant Bcl-2 protein was capable of channel formation, measured by KCl efflux from preloaded vesicles [138]. By contrast, Antonsson and coworkers [139] found Bax to be capable of releasing carboxyfluorescein from liposomes at neutral and acid pH, which could be blocked by Bcl-2 at pH 7. On the other hand, at acid pH even Bcl-2 formed pores through which the dye could diffuse out of the vesicles. Are these pores related to the PT? If the description is confusing to the reader, he/she is not alone. Many phenomena and players remain to be coordinated and explained in this field.

It is entirely plausible that "turning off the power" is one of the major events in a cell determined to commit suicide. A study of respiration-deficient mammalian cell-mutants has shown that such cells die within a day when they are switched from a high-glucose medium to medium containing galactose. In the absence of oxidative phosphorylation cells are sustained by a high rate of glycolysis [24] which is not possible with galactose (see Section 7.1.2). On the other hand, several experiments in cell free systems suggest a more complex picture. Isolated nuclei can be induced to undergo apoptosis when exposed to cytoplasmic extracts from cells in the process of apoptosis. Controversy existed about whether such extracts can be free of mitochondria, or whether enrichment with mitochondria accelerates the process. When shown to be required, only mitochondria that have made the permeability transition (e.g., by treatment *in vitro* with atractyloside), and not healthy mitochondria, were claimed to induce nuclear apoptosis. It has been suggested that such mitochondria release an apoptogenic protein (apoptosis inducing factor [AIF]). This protein has been characterized as a 50-kDa intermembrane protein, with protease or protease activating activity but not nuclease activity, which can induce apoptosis in isolated nuclei even more efficiently than ceramide, another mitochondrial constituent apparently released by the PT. The release of AIF is also inhibited when Bcl-2 is exerting its protective function in transfected cells [134].

### 7.4.3 Apoptosis and Cytochrome c

More recently, *in vitro* studies of characteristic events associated with apoptosis (DNA fragmentation in isolated nuclei, cleavage of CPP32 precursor, the mammalian equivalent of the ced-3 protease) has shed more light on mitochondrial involvement. In an experimental system in which apoptosis can be initiated by addition of dATP, the fractionation of the cytoplasm revealed the requirement of a protein identified as cytochrome c. The need for cytochrome c was further demonstrated by immunodepletion and reconstitution experiments [140]. These and subsequent studies have suggested that the cytochrome c is involved in triggering the cleavage and activation of DEVD-specific caspases. If cytochrome c release is an event preceding the caspase cascade, then a role for Bcl-2 in preventing cytochrome c release can be postulated, and *in vitro* the addition of exogenous cytochrome c is expected to bypass the pro-



protective effect of Bcl-2. Support for these ideas was derived from another cell-free apoptosis system based on *Xenopus* egg extracts [141]. In such a system the requirement for mitochondria has been established, and the timing of cytochrome c release has been correlated with the caspase activation. Addition of exogenous Bcl-2 prevented cytochrome c release and caspase activation as well as other apoptotic events. Kluck and associates also demonstrated that Bcl-2 must interact directly with the mitochondria to prevent cytochrome c release. Furthermore, in this cell free system the mitochondrial membrane potential was maintained throughout the progress of apoptosis, regardless of whether Bcl-2 was added or not. Thus, it was argued that cytochrome c release in this system is not associated with the permeability transition, PT. Presumably, the AIF, a 50-kDa protease, is not involved in this system, if its release does depend on the PT.

Thus, while some authors have emphasized the maintenance of  $\Delta\Psi_m$  and the protection by Bcl-2 against the permeability transition, protection against cytochrome c release from still energized mitochondria is an alternate mechanism. An important experiment has shown that Bcl-2 can block apoptosis even in cells which lack mitochondrial DNA and are unable to carry out oxidative phosphorylation [142]. This is not to say, however, that they lack a membrane potential, since such cells, and mutant cells completely defective in mitochondrial protein synthesis [25,27] are perfectly capable of mitochondrial protein import. The release of cytochrome c from such mtDNA-less cells could account for these observations.

#### 7.4.4 Protein-Protein Interactions

The final word on Bcl-2, mitochondria and cytochrome c has not been spoken [142a]. There are now reports that the Bcl-x<sub>L</sub> proteins protects cells by binding to cytochrome c, and that Bcl-x<sub>S</sub> prevents the interaction of Bcl-x<sub>L</sub> with cytochrome c [143]. Another group reports on Fas-mediated apoptosis in Jurkat cells that cytochrome c is inactivated by a heat-labile cytochrome c-inactivating factor of apoptosis (CIFA). Apoptosis in these cells is associated with making the outer mitochondrial membrane permeable to CIFA, and inactivation of the cytochrome c interrupts electron transport. Bcl-2 is claimed to protect mitochondria against CIFA [144]. One is left with the impression that there is much to be resolved, if a simple pathway is to account for these diverse observations, or that there are numerous pathways activated in different cells under different circumstances.

Reactive oxygen species can be produced at increasing rate in defective mitochondria, but also on the ER membrane and the outer nuclear membrane (a continuation of the ER membrane). They can also be produced by treatment of cells with H<sub>2</sub>O<sub>2</sub> or *t*-butylhydroperoxide which generate O<sub>2</sub><sup>-</sup> and at low concentrations kill cells by an apoptotic pathway. Bcl-2 can protect cells against such agents, and therefore it has been proposed that Bcl-2 either inhibits free radical formation or protects against a decrease in intracellular glutathione (GSH). Neither claim can be supported by any identified biochemical activity of Bcl-2, and its role may be indirect.

It has been shown convincingly that Bcl-2 and related family members can form heterodimers with death promoters such as the Bax and Bad proteins. Two protein domains involved in the interaction have been identified by site-directed mutagenesis,

and sequence comparisons in the Bcl-2 family show conservation in these domains [133]. One of these regions comprises approximately 24 amino acids near the N-terminal, which are predicted to form an  $\alpha$ -helical conformation, with five residues clustered on one face of this helix and thus implicated in protein-protein contacts in homo- or heterodimers. Bax peptides can also form homodimers, and a model has been proposed where cell survival and cell death is determined by the relative abundance of the various family members such that excess Bax/Bax homodimers cause cell death. In this model Bcl-2 plays the relatively simple role of titrating excess Bax molecules to form Bcl-2/Bax heterodimers which are not neutral. The biological activity of Bax homodimers remains obscure.

In general, Bcl-2 seems to exert an inhibitory effect on the activities of the ICE protease/caspase family. This relationship was first deduced from the relationship of the *ced-3* (ICE homologue) and *ced-9* (Bcl-2 homologue) genes in *C. elegans* development, and later confirmed by an experiment in rat fibroblasts where overexpression of ICE leads to apoptosis which can be prevented by Bcl-2. In at least one system the presence of an ordered cell death pathway has been established in which the Bcl-2 protein acts upstream of the ICE family protease actions [145]. The nature of the interaction among the numerous Bcl family members with the various proteases has not been clarified, but it is likely to be an indirect one.

The examination of mice in which either the Bcl-2, the Bcl-x or the Bax genes were knocked out has yielded a complex picture. Bcl-2  $-/-$  mice are born as viable pups but die at a few weeks of age. Bcl-x  $-/-$  mice die around day 13 of embryonic development, due to massive cell death in the central nervous system among other tissues. Bax  $-/-$  mice appear healthy, with lymphoid hyperplasia and sterility of males due to lack of sperm cell production. Overall such studies support a lineage-specific role for these genes, but details remain to be clarified, and for the moment no significant relationship to mitochondrial function or activity can be seen from such studies in intact mice.

A new role for Bcl-2 on the outer mitochondrial membrane has recently been suggested by Wang and coworkers [146], building on information about two additional conserved domains in the Bcl-2 family of proteins. While BH1 and BH2 are important for heterodimer formation with other anti- and proapoptotic members, the BH4 domain near the N terminus appears to be responsible for an interaction with the Raf-1 protein kinase. This kinase has been well-studied in connection with the growth factor receptor-mediated pathway of signal transduction, including the p21<sup>ras</sup> proteins at the plasma membrane. In these experiments it was demonstrated that targeting of Raf-1 to mitochondria, either by means of binding to Bcl-2, or directly by producing a Raf-1 protein with a targeting sequence to the outer membrane could enhance the protection against an apoptotic stimulus. Thus, while Ras promotes association of Raf-1 with the plasma membrane, Bcl-2 appears to promote the association of this kinase with the mitochondrial membrane. Moreover, this difference in intracellular location of Raf-1 also leads to a different spectrum of proteins being phosphorylated. Among the novel protein becoming phosphorylated by the mitochondria associated Raf-1 is the Bad protein, another Bcl-2 family member.

The discussion so far has raised many unanswered questions about apoptosis in general and about the role of mitochondria specifically. Bcl-2 levels and its interac-

tion with its relatives appear to determine the sensitivity of cells to apoptotic stimuli. The mitochondrial permeability transition is a characteristic and probably fatal event in the overall mechanism of programmed cell death. The majority of Bcl-2 peptides are targeted to the outer mitochondrial membrane. What is unclear at this time is how these facts are related in direct or indirect ways. Is Bcl-2 located at or responsible for the PT pore formation, or is this megapore assembled as a result of phosphorylation reactions promoted by the recruitment of the Raf-1 kinase to the mitochondrial outer surface? Is the permeability transition and the cessation of normal mitochondrial activity an event proceeding in parallel with protease and nuclease activation and the hydrolysis of a variety of macromolecules, or is there a more causal connection? Is the release of cytochrome c and AIF an essential event with a signaling function to the nucleus? It is easier to knock out the Bcl-2 gene in a mouse than to answer these important questions. What can we learn at a molecular level from the fact that an apparently healthy Bcl-2  $-/-$  mouse dies after a few weeks?

## 7.5 FUNGAL SENESCENCE

Many fungi appear to be able to divide mitotically for an almost unlimited period of time, growing into large clones whose size may be limited only by physical parameters. In the wild, *Armillaria bulbosa* colonies in excess of 0.5 km diameter have been found. On the other hand, some strains in the laboratory exhibit the phenomenon of senescence, that is, they cannot be serially transferred indefinitely in culture. In a few prominent cases the phenomenon has been investigated genetically. Since mitochondrial genomes were found to play a large role in the process, it has been tempting to draw parallels with aging in humans, but as our understanding has advanced, the resemblance is only at the physiological level: the inability of cells to undergo further mitotic divisions and a generalized cellular decay at the end. The best-studied examples have been expertly reviewed by Griffiths [147,148], and some salient features are appropriate for the present chapter. Although nuclear genes can play a role, a common theme is that senescence in fungi is associated with cytoplasmic inheritance. A triggering event appears to be an alteration in the mitochondrial genome, which eventually leads to a severe mitochondrial degeneration and dysfunction and hence senescence.

The existence of mitochondrial plasmids in many organisms, and fungi in particular, has already been discussed in Section 4.1.6. Even at relatively high copy numbers they may act as relatively innocuous molecular parasites, expressing functions primarily devoted to their own autonomous replication. In *Neurospora crassa* and *Neurospora intermedia* plasmids isolated from wild strains have been shown to be responsible for senescence. The kalilo plasmid (~9 kb) isolated originally in Hawaii, causes *N. intermedia* to stop growing, and in the terminal stages abnormalities in cytochrome spectra and content, deficiencies in large ribosomal subunits, and loss of cyanide-sensitive respiration are observed. It is now clear that the plasmid can insert at one of several locations in the mitochondrial DNA. There may be more than one insertion in the process of senescence, and a mtDNA population may evolve until at death a predominant insert is observed. A similar picture is seen with the maranhar

plasmid isolated in a *N. crassa* strain in India, and other plasmids found in a large sample of natural isolates [149]. While there appears to be no doubt that the kalilo and maranhar plasmids are the responsible agents, other plasmids in *Neurospora* are neutral. The challenge therefore is to understand why some plasmids can and do insert themselves in the mtDNA. According to Griffiths, the "initial insertion event is probably best viewed as a mistake." The mistake, however, is irreversible and ultimately lethal. Does it have any biological significance, especially outside of the laboratory?

Historically, senescence in the fungus *Podospora anserina* was one of the first to attract attention, since all natural isolates died, but they each exhibited distinct and reproducible life spans. Cytoplasmic inheritance was also observed, but heterologous, extragenomic mitochondrial plasmids cannot be detected. Instead, as senescence progresses, circular DNA molecules derived from the mtDNA are observed. The triggering event is unclear, but the net effect is that one of several different segments of mtDNA (labeled  $\alpha$ ,  $\beta$ ,  $\gamma$ , etc.) becomes excised, associated as head-to-tail multimers, and circularized to form a series of senDNAs. At the same time, intact mtDNA molecules disappear. The most common sequence is found in  $\alpha$ -senDNA, which consists of monomers identical in sequence to the first intron of the CO1 gene (cytochrome oxidase). It may be significant that this group II intron encodes a protein with homology to reverse transcriptase, but generalizations of the mechanisms for senescence in *Podospora anserina* must include an explanation for the  $\beta$ -,  $\gamma$ -,  $\delta$ -, and other senDNAs which are not restricted to introns, and do not encode obvious functions [147]. Curiously, a senescing culture can be rejuvenated by treatment with ethidium bromide, as if the intercalator were capable of selecting for wild-type mtDNA molecules, since the sen plasmid present at the time of addition is eliminated. When ethidium bromide is removed again, senescence recurs in association with the appearance of a different senDNA type.

Numerous studies have sought to identify strains with different life spans, and to identify nuclear or mitochondrial mutations responsible for this variation. Details can be found in the reviews by Griffiths [147,148]. For example, a short-lived strain dies only on solid medium, but not in liquid culture. After repeated transfers in liquid cultures, immortal cultures could be obtained. These were female sterile and incapable of transmitting the immortality as males. Mitochondrial mutations were likely responsible for the altered phenotype.

In conclusion, senescence in *Podospora anserina* can be linked to mitochondrial DNA instability, but here also the triggering mechanism, the transition from the non-senescent (juvenile) state to the senescent state, remains to be elucidated. As in the case of the *Neurospora* plasmids, the true biological significance of the phenomenon of this induced aging remains another puzzle.

## 7.6 CYTOPLASMIC MALE STERILITY IN PLANTS

Cytoplasmic male sterility (CMS) has been presented briefly in a different context in Section 4.1.6. The integration of specific plasmids into mtDNA can cause CMS in maize [150]. Another genetic mechanism, independent of the existence of extragenomic plasmids, will be illustrated here with a select number of examples. It should

be noted that the phenomenon has been observed in more than 150 plant species. The involvement of the mitochondrial genome in all cases points to a crucial role of mitochondrial function(s) in the development of the male reproductive system and pollen formation in particular.

The exceptionally large mitochondrial genomes in plants have already been discussed (Section 4.1.3) in terms of their high recombination rate, thought to include a population of large circular mtDNA molecules, each representing a portion of the genome. Specific "recombination repeat" sequences typically greater than 2 kb are responsible for homologous recombinations capable of permuting the genome without mutating the coding regions. Occasionally, illegitimate (?) recombination events involving much shorter repeat sequences can occur, and they may be responsible for the evolutionary changes in the plant mt genomes. Even more rarely, such recombination events can create new chimeric genes responsible for CMS. The search for such new sequence arrangements in the large plant mtDNA is a formidable task.

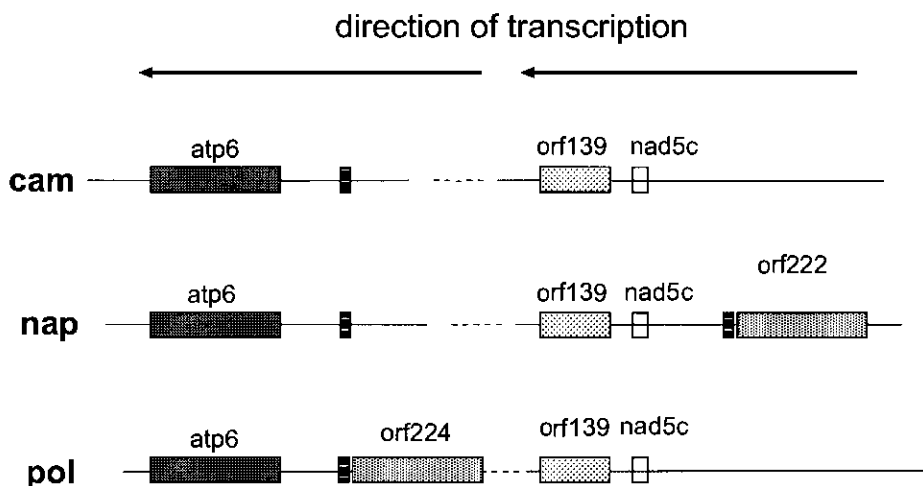
L'Homme and colleagues have thrown considerable light on the situation in the oilseed rape (canola) species *Brassica napus* [151], and the results may exemplify the general nature of the genetic changes associated with CMS. The fertile *B. napus* plant has a cytoplasm (mitochondrial genome) referred to as *cam*; two infertile strains (male sterility) have cytoplasms (mitochondrial genomes), *nap* and *pol*. The *cam* cytoplasm may have originated from the diploid *B. campestris* progenitor of the amphidiploid *B. napus*. The *nap* cytoplasm makes plants male-sterile only when certain nuclear *Rf*'s (restorer of fertility) are missing, but the *pol* cytoplasm makes most *B. napus* cultivars male sterile. The whole mtDNA sequence is not yet available, but many segments containing known genes have been identified and physical maps of restriction fragments are available. The task was to find differences between the *cam*, *nap*, and *pol* mt genomes.

Over the past five years several lines of evidence have pointed at the vicinity of the *atp6* gene as the region differing in the *cam*, *pol*, and *nap* mtDNA. Detailed restriction mapping, sequencing, and the analysis of chimeric transcripts found in the various strains has yielded a picture of the arrangement of sequences in the three mtDNAs which confirms the postulated differences at this location and is consistent with the observed CMS phenotypes [151]. In *pol* cytoplasms the *atp6* gene is cotranscribed with a chimeric gene *orf224*. The *orf224* sequence is contained within 4.5-kb insert upstream of the *atp6* gene which is absent in the *cam* mtDNA. A very similar copy of this 4.5-kb insert was also found in the *nap* mtDNA near the *atp6* locus, but it was further upstream of *atp6*, and only monocistronic *atp6* transcripts were found in this cytoplasm. Detailed sequencing of the 4.5-kb insert in the *nap* mtDNA revealed an ORF with the same initiation and termination codons as in *orf224*, but with only 85% sequence similarity and two fewer codons. It was designated *orf222*. Transcripts detected with the *orf222* probe were larger than expected, and further analysis showed co-transcription with two additional ORFs, one of which could be identified as the trans-spliced exon c of the *nad5* gene, while the other (*orf139*) remains unknown.

Details of this painstaking analysis can be found in the original publication [151], but the reader should come away with a sense of the types of rearrangements en-

countered in plant mitochondrial genomes which can lead to a CMS phenotype. Needless to say, L'Homme and colleagues had to prove that expression of the *orf222/nad5c/orf139* is indeed responsible for the male sterility. This was accomplished by probing Northern blots of RNA from male sterile, fertile, and restored cytoplasm with a variety of probes, including *orf222*, *nad5c*, and sequences from sites where a previous analysis by Palmer and Herbon had shown differences between *nap* and *cam* mtDNAs. Over 40% of the *Brassica* mitochondrial genome were surveyed, with the result that the *orf222/nad5c/orf139* region is the only one affected by nuclear restoration. The result is consistent with the general conclusion that all CMS phenotypes can be correlated with differences in the organization, rearrangements and possibly small deletions of plant mitochondrial genomes [153]. The transposition of the 4.5-kb fragment may have involved a 10-bp repeat found upstream of *atp6* and at the downstream end of *orf222* and *orf224* (see Figure 7.3).

The fact that illegitimate recombination between plant mt genomes can create chimeric genes and novel transcripts, may no longer surprise. However, the study by L'Homme and colleagues also indicated that a simple transposition of the 4.5-kb fragment is not sufficient, and further sequence changes had to accumulate. Presumably there must then also exist a mechanism to convert the plant to a homoplasmic condition, or at least to a heteroplasmic state where the mutation is dominant. One study in which this question was specifically addressed found a very low level of the "wild-type" (fertile) mtDNA in the sterile cytoplasm of sunflower. However,



**Figure 7.3** Schematic illustration of rearrangements in the mt genome of *Brassica napus* associated with cytoplasmic male sterility [151]. In the male-sterile *nap* and *pol* mt genomes insertions have occurred upstream of the *atp6* locus, with reference to the same locus in the male-fertile *cam* genome. The shaded boxes represent open reading frames; *orf224* and *orf222* share a 10 bp repeat at the 3' end (small black box), and otherwise have 85% nucleotide sequence similarity. The trans-spliced exon *nad5c* is indicated by the small open box. The dotted lines represent unique sequences.

the analysis of heteroplasmy is potentially more complicated for plant mtDNAs because of the frequent recombinations generating aberrant recombinant molecules (sublimons) at substoichiometric, low levels [see Reference 154 for discussion and references]. Finally, the biologically most interesting question is: which genes are affected, and how does the disruption of a mitochondrial gene specifically cause male sterility, that is, abnormal or aborted anther or pollen development? Analyses in this and other plants lead to the hypothesis that the CMS phenotype is the direct result of expression of the chimeric genes, rather than due to an effect on transcription, splicing or translation of the normal genes. Nevertheless, no significant sequence homology has been detected in CMS-associated genes from different plant species which would point at a particular protein or function being required. Although *orf224* and *orf222* have extensive homology, morphological, developmental, and genetic differences distinguish the two *Brassica* CMS systems. For example, the nuclear *Rf* genes restoring male fertility are different for *pol* and *nap*. The diversity of mechanisms giving rise to CMS is also illustrated by the existence of multiple types of CMS in maize and other plants [155].

Results which are closer to answering questions raised above come from a study of male sterility in the sunflower. In this case as well an unusual ORF (*orf522*) had been created and associated with CMS. It is long enough to encode a 15-kDa peptide, and this peptide had been identified in sterile lines. In a recent study Moneger and colleagues [154,156,157] showed that a dominant nuclear restorer gene can suppress this 15 kDa peptide significantly in a tissue-specific manner, that is, the reduction is observed only in male florets. Furthermore, run-on transcription experiments with isolated mitochondria from this specific tissue and comparisons with steady state levels of the transcript in sterile and restored hybrid lines strongly suggest that the nuclear *Rf* product regulated *orf522* transcript levels posttranscriptionally.

The 15 kDa ORF522 peptide also shows no homology with a 13-kDa mitochondrial peptide (URF13) from maize CMS-T, or the 25 kDa mitochondrial peptide from the *pcf* locus in petunia, both of which have been implicated in the mechanism leading to CMS in these plants. (One should be reminded again that protein coding genes in plant mitochondria are edited, and the nature of the specific base change(s) must be known before sequence comparisons can be made). However, these proteins all appear to be relatively hydrophobic, and there is speculation that their expression and integration into the mitochondrial inner membrane may interfere with OXPHOS.

Several scattered observations are relevant for explaining specifically male sterility. First, a 20- to 40-fold increase in the relative number of mitochondria has been observed in maize anthers [158,159]. Second, the levels of transcripts of a select number of mitochondrial genes (*cob*, *atp6*, *atp9*) have been determined to be elevated in microspores compared to leaves in maize [160]. Finally, isolated mitochondria from different tissues of maize and sugar beet synthesize organ-specific polypeptides [161,162]. Thus, mitochondrial activity and function clearly appear to be significant factors in pollen formation. In other tissues a diminished capacity for OXPHOS due to mtDNA rearrangements may be less influential, or OXPHOS may not even be affected if the CMS-associated ORF is not expressed.

Another advance in the quest for understanding the molecular basis for CMS was added by an elegant study from MacKenzie's laboratory [163]. In CMS common bean rearrangements in the mtDNA have created two new ORFs, *orf239* and *orf98*, and a 27-kDa peptide encoded by *orf239* was found exclusively in the reproductive tissues. This and other evidence made the *orf239* peptide a prime suspect for a primary role in CMS, and to prove it, the authors expressed the *orf239* sequence after transformation into tobacco plants. The gene was engineered to express the 27-kDa protein with or without a mitochondrial targeting sequence; specific promoters were attached to restrict the expression of the chimeric genes to developing microspores. The perhaps unexpected results were that male sterility and defective pollen development were observed regardless of whether the mitochondrial targeting sequence was present. The presence of a cryptic targeting sequence in the *orf239* peptide was excluded on theoretical grounds, and the conclusion was that pollen development could be interrupted by a protein expressed in the cytoplasm. Immunoelectron microscopy was used to localize the protein exclusively in developing microspores, and there it appeared to be in the cell wall of developmentally arrested microspores. The transgene was dominant with incomplete penetrance, possibly due to the less-than-perfect timing of expression from the heterologous promoter.

Two major results emerge from this study. First, a CMS-related gene product may act without compromising mitochondrial function. Second, in the original CMS common bean the 27-kDa protein is made inside the mitochondria, and hence it has to be exported to interact with the extramitochondrial target. The next stage will address whether export is from intact mitochondria by a novel pathway, or whether the protein can be dumped into the cytosol by mitochondrial lysis.

In summary, the relationship between CMS and mitochondria is a fascinating subject with many challenges ahead. And while mitochondrial genes may play a central role in this phenomenon, it cannot be explained by the action of a single gene product. Further study, with CMS as a focus, will undoubtedly shed significant new light on the development of the male reproductive system in plants in general.

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# 8

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## MITOCHONDRIAL DNA POLYMORPHISMS AND ANTHROPOLOGY

### 8.1 INTRODUCTION

In 1987, Cann, Stoneking, and Wilson [1] published a paper on the analysis by restriction mapping of mtDNAs from 147 people, drawn from five widely spaced geographic populations (34 Asians, 21 Australian aboriginals, 26 aboriginal New Guineans, 46 Caucasians, and 20 Africans). The scientific impact of this paper was enormous. The application of methods from molecular genetics to the study of primate and human evolution, with a focus on mitochondrial DNA, had been suggested by Brown [2], and several publications by Brown, Wilson, and colleagues had preceded the landmark 1987 paper [3–5]. However, the large number and diversity of people represented in this paper provided convincing evidence for the potential strength of this approach in addressing questions about the relatively recent human evolution, about the origins of modern humans and about the migratory patterns of our ancestors. The abstract of the paper contained the eye-catching and provocative statement: “All these mitochondrial DNAs stem from one woman who is postulated to have lived about 200,000 years ago, probably in Africa.” Unfortunately, the popular press took this statement literally, and the misconception arose that she was the single ancestor of us all. The catchy headlines derived from this study certainly promoted interest in this field and popularized human evolution among the molecular biologists who can relate more easily to a DNA sequence than to a jawbone.

In the past decade the analyses have not only been vastly refined by the use of many more restriction enzymes, the application of the powerful polymerase chain reaction, and actual sequencing of defined regions, but human mtDNAs from the remotest areas of the globe have been analyzed, and one can only imagine what it would be like to explain to a Chukchi from northeasternmost Siberia or a Yanomama from

South America why their mtDNA is of such interest and how it proves that ultimately they all had descended from a mother in Africa.

The biological and molecular basis for the value and fruitfulness of mitochondrial DNA sequence variations is derived from three facts. There is a strictly maternal mode of inheritance, hence no scrambling of information by recombination during sexual reproduction. The rate of evolution of mtDNA is estimated to be an order of magnitude greater than that of nuclear DNA, thus providing resolution on a time scale that is appropriate for human evolution: a few thousand years is sufficient time to generate informative variants. Finally, mtDNA represents a distinct molecule that has been completely sequenced. It is abundant in cells (high copy number), and therefore easily accessible for experiments.

The maternal mode of inheritance is an important consideration. There is universal agreement that nearly 100% of mtDNA in an individual is derived from the mother's egg, and no complications are expected in the analysis of a pedigree of three or more generations. However, phylogeny on an evolutionary time scale could well be influenced by the rare transmission of mtDNA from the paternal side, and a challenge of the current dogma was issued recently from a broad consideration of fertilization and the fate of the sperm mitochondria [6], and also from some very well-controlled studies with interspecies hybrid mice [7]. Since there was evidence for biparental inheritance in interspecies hybrids, it became urgent to prove that this was the exception rather than the rule, and some such proof has been provided recently [8]. There is no argument over the observation that sperm mitochondria are found in the zygote shortly after fertilization. The most recent studies suggest, however, that paternal mtDNA is specifically eliminated in intraspecies crosses by a mechanism yet to be elucidated. In interspecies crosses, by contrast, the mechanism apparently fails to recognize the paternal mtDNA, and therefore it escapes destruction and elimination.

The accumulation of base changes in mtDNA occurs on a time scale sufficiently short to generate polymorphisms within populations of the same broad ethnic category (e.g., Caucasians); there are polymorphisms distinguishing the various human population groups; and there are polymorphisms within a single individual, where we refer to them as heteroplasmy. Heteroplasmy in relation to mitochondrial diseases has been discussed in Chapter 7. In an apparent paradox, most individuals are homoplasmic. Homoplasmy is either the result of a severe "bottleneck" in oocyte development (see Chapter 7), or more apparent than real, depending on the sensitivity with which the analysis or search for heteroplasmy is carried out [9].

## 8.2 HUMAN EVOLUTION

While sequencing the mtDNAs of large numbers of diverse humans provides hard and undisputed data, the interpretation of the data has created a great deal of controversy. At first it looks deceptively simple. In their original eye-opening paper, Wilson and his students simply compared the number of mutations in various peoples [1]. A population that has been in existence and in place for a long time should, upon sampling, reveal a large diversity of mitochondrial genomes, assuming that the molecular clock



(nucleotide changes per unit time or per generation) was ticking away steadily. A population established by a small number of founders only recently would be expected to be relatively homogeneous with respect to mtDNA sequences. This was indeed found: Africans had by far the greatest diversity, a finding which was interpreted in terms of a mitochondrial Eve out-of-Africa. A second aspect of the same study was to use estimated rates of the molecular clock to deduce that the total number of mutations observed in the African population would require somewhere between 140,000 and 280,000 years. Criticisms soon appeared, related to several methodological details of the study. Restriction analysis is a crude way of sampling a sequence, and with a small sample size valuable information could be missed or misinterpreted. African-Americans had been used to represent Africans, with a possibility of bias. Finally, the formal methodology to build the phylogenetic tree was questioned.

In time, many of the criticisms were refuted. The sample size and the number of representatives from each geographic region could be increased significantly, including contributions from other groups of researchers. The sequence analysis could be improved by the inclusion of more restriction enzymes and even complete sequences. The mtDNA segment favored in these studies is the control region, a noncoding region containing promoter elements and origins of DNA replication (see Chapter 4) which evolves particularly quickly. The massive amount of data was analyzed with a newly developed computer program called PAUP (Phylogenetic Analysis Using Parsimony). Such a program is designed to generate the most parsimonious phylogenetic trees, and this is where the controversy starts. When Wilson and his colleagues [10] followed their original 1987 paper with another much more detailed analysis in 1991, they concluded that our common mtDNA ancestor indeed lived in Africa about 200,000 years ago. Lost in the detail, but discovered by a subsequent critic, was the untenable splitting of the 25 !Kung bushmen of Africa on the deepest branches of the tree, in the face of their seemingly obvious relatedness. Recalculations showed that the computer program had not succeeded in finding the most parsimonious tree, and that there is no absolutely unique and most parsimonious solution (at least with a limited computation time). Even with the best solutions, the tree has no obvious roots, or roots pointing unambiguously to Africa.

A recent reexamination of the mtDNA data of Cann [1] and of Vigilant and colleagues [10] was attempted by Wills [11]. This highly technical paper introduces a method referred to as "topiary pruning," a term that describes the attempt to reveal the true shape of an ornamental topiary tree by removing the obscuring "new growth." It is argued that a phylogenetic tree may have similar new growth, which can obscure significant branches. The "new growth" on a phylogenetic tree may be the result of recent, repeated mutations in different lineages at hypermutable sites. The pruning produces datasets with a reduced number of taxa, and this simplification in turn makes the datasets more amenable to analysis by a variety of statistical methods. A combination of such methods (pruning, weighting, bootstrapping) is expected to yield a tree that is closer to the "true" tree. While Wills is careful to state that the African origin of the human mitochondrial tree is not proved by this approach, the interpretation of the pruned tree adds strength to the argument in favor of an African origin.

There are additional disputes about the calibration of the clock. Is the rate of change the same at all sites where changes have been observed? Do noncoding re-

gions (D-loop) change more rapidly than coding regions? Are the changes in the third positions of the codons occurring at a faster rate? What about the relative rates of transitions vs transversions in a DNA sequence? The challenge was taken up by Hasegawa and colleagues [12] to predict a more accurate time scale for human mtDNA, making the additional assumption that the divergence of humans from our closest primate ancestor, the chimpanzee, was about 4 million years ago. The emergence of modern humans out of Africa was moved to an even more recent date:  $89,000 \pm 69,000$  years ago. Such a result also put the authors squarely at odds with the multiregional hypothesis of modern human origin which has gained adherents in recent years. Uncertainties and controversies related to the mutation rate in mitochondria have by no means been resolved to everyone's satisfaction, and as a result the estimates for the age of the mitochondrial Eve range from less than 100,000 years to almost a million years (see below).

After the African mitochondrial Eve was declared "wounded but not dead yet" [13] in 1992, based on the ambiguity surrounding the construction of phylogenetic trees with mtDNA sequences, supporters of the hypothesis have assembled independent support. The controversy has at times been heated. An attempt by a respected expert to debunk the "myth of Eve" [14] was followed a few issues later by a rebuttal by another expert accusing the first of perpetuating another myth [15]. As Ayala states succinctly, the coalescence of the mtDNA sequences from over 100 ethnically diverse individuals to one ancestral sequence, that of the "mitochondrial Eve," is a property of all gene genealogies, but does not mean that all humans descended from a single mother. While Wilson and his colleagues probably also did not mean to be taken literally, the statement had an attention-catching appeal, and the popular press made the most of it. If not a single mother, there is still the question about the size of the pool of humans from which the present-day human groups are descended. The current controversy therefore centers around two or three problems. Where is the mtDNA genealogical tree rooted? Evidence points strongly toward Africa, but it is not yet conclusive. The second question is concerned with the existence of a bottleneck, and the size of the bottleneck of those individuals who are the ancestors of all modern humans. Finally, there may have been multiple bottlenecks with subsequent mixing of the surviving populations. The arguments about multiregional models require extensive migrations and interbreeding between populations to account for a single gene pool [14].

Ingenious independent analyses have strengthened the out-of-Africa hypothesis. To establish the true root of the phylogenetic tree, the true human mtDNA ancestor, one must choose an outgroup sequence from a different species more ancient than humans, but still closely related. The chimpanzee mtDNA has served such a purpose, but in the control region used routinely the rate of change may be too fast and too extreme. The creation of multiple and parallel sequence changes can complicate and obscure the analysis. A novel finding was exploited elegantly by Zischler and coworkers [16] to identify a superior outgroup for rooting the human mtDNA tree. Following the original discovery of Fukuda and coworkers [17], several investigators have confirmed that the human nuclear genome contains several hundred copies of sequence fragments closely resembling mtDNA. Such pseudogenes have also been found in other primates and rodents, and their distribution and sequence alterations make them useful molecular fossils in the nucleus [18]. Zischler and colleagues [16] specifically

searched for and found a nuclear copy of the human mitochondrial D loop which had been transferred to the nucleus after the divergence of humans from the chimpanzee, but before the "growth" of the human mitochondrial phylogenetic tree. The nuclear D-loop sequence became a superior outgroup for rooting the tree in Africa [19].

The mtDNA data assembled by many groups, prominently among them the group at Emory University, and Stoneking and colleagues at Pennsylvania State University, generally supported the following scenario for the expansion of *Homo sapiens* around the globe [20]: The migration started out of Africa about 100,000 to 200,000 years ago. It split somewhere in the Middle East into a group headed for central and northern Europe (~50,000 years ago), and another group headed toward southern and southeast Asia. Migrations from present-day China via the Indonesian Archipelago toward New Guinea and Australia also occurred a long time ago. Somewhat later the populations expanded north along the eastern rim of Asia, and eventually groups reached the Bering Strait to cross into North America. There may have been several crossings of the Bering Strait (see below). Expansion(s) from Alaska to the south populated North America, Central America, and South America (in that order). Quite recently in human history another wave of expansion occurred from the islands surrounding New Guinea and North Australia into the northern Pacific (Polynesia and Hawaii).

Of course, many of these deductions were not completely new. Data from paleo-anthropology and archeology had already been used to make broadly similar deductions. Linguistic comparisons have also contributed greatly. Nevertheless, mtDNA sequence data can often provide detailed and convincing support. For example, a 9 bp deletion in the small intergenic region between COXII and tRNA<sup>Lys</sup> is believed to have arisen in Asia about 50,000 years ago. Redd and associates [21] have traced a specific set of control region mutations ("the Polynesian motif") associated with the 9-bp deletion to a Taiwan origin (about 6,000–8000 years ago), and from there across the Philippines, Indonesia, along the coast of New Guinea, and finally into Polynesia. These molecular data are in excellent agreement with the postulate of a southeast Asian origin of Polynesians based on archeological and linguistic evidence.

The contention about rooting the human phylogenetic tree may have approached a consensus, or at least the African root has acquired the support of the majority. It is yet another issue to date the period of the existence of the mitochondrial Eve. Determining the age of Eve is important, because an accurate estimate of the time span to the present serves in arguments about other theories, specifically the multiple origins theory. Since the fossil record reveals the existence of *Homo erectus* as long as at least a million years ago, and such evidence is found in Europe, the Middle East, and Asia, one is forced to conclude from the genetic evidence that the modern humans (our ancestors) displaced all of these other human populations completely without any detectable genetic exchange. If the lowest estimate for the age of the mitochondrial Eve is accepted (<100,000 years), this scenario would be quite astonishing. On the other hand, if the mitochondrial Eve lived more than 500,000 years ago, a new set of questions and theories can be entertained about multiple origins, shifts in the composition of the human gene pool, and selective pressures which might have contributed to the unusual evolutionary pattern. A thoughtful reexamination of existing datasets by Wills [22] comes to the conclusion that Eve may be between 436,000 and 806,000

years old, an estimate which is also very dependent on the estimate of the time of the split between the human and chimpanzee lineages.

mtDNA sequence analysis has been perfected to the level where analyses have become possible with samples from a 4000-year-old mummy of an Egyptian priest, a 7000-year-old human brain from a Florida peat bog, and, if the bones are ever released for analysis before they are reburied, there are paleobiologists waiting to obtain samples from a skeleton found on the northwest Washington coast thought to be 9000 years old.

One of the most impressive achievements in the study of human evolution by the study of human DNA sequences was reported by Krings and colleagues in 1997 [23]. The group in Munich, with independent confirmation by a group in Pennsylvania, reported mtDNA sequences from a Neandertal person. The Neandertals were a race of humans roaming through Europe and Western Asia from about 300,000 to 30,000 years ago. Considerable discussion had centered around the question whether *Homo neandertalensis* was a distinct and separate species from *Homo sapiens*. Their range overlapped in Europe during the more recent millenia, and the possibility of a mixing of the gene pools by interbreeding had not been convincingly excluded. In the landmark paper published by the S. Pääbo and M. Stoneking groups the definite conclusion was reached that the Neandertals went extinct, leaving no trace of their mtDNA in modern humans. Concentrating on the D-loop region, sequence comparisons and phylogenetic analyses were made, including a large number of representatives of modern humans as well as the chimpanzee. The divergence of the Neandertal from modern humans was estimated from the mtDNA analyses to have occurred about 500,000 years ago, in satisfactory agreement with other estimates based on the archeological record.

The paper is a landmark not only for its conclusions, but it probably also sets the standard for future studies of ancient DNA. The DNA was retrieved from a fossilized bone of an individual living between 100,000 and 30,000 years ago. As discussed in more detail in this paper and elsewhere, measurements of amino acid racemization can give reliable estimates about preservation and the feasibility of obtaining sufficient DNA from a fossil specimen, and the Neandertal bone may be near the limit of what constitutes a useful source. The quantitative considerations also indicate that only mtDNA sequences can be expected to be recovered for PCR-based amplification because it is several orders more abundant than nuclear DNA. In a commentary on this paper in the same issue of *Cell*, T. Lindahl [24] discusses the "fiasco of DNA from insects in amber," with reference to studies suggesting that amber is a very poor material for the preservation of DNA (but not of the insect exoskeleton). Thus, claims of DNA amplification from million-year-old insects in amber may continue to stimulate Hollywood, but they cannot stand up to rigorous scrutiny. Because such skepticism is clearly indicated, the Neandertal mtDNA paper went to extraordinary lengths to include controls, and to obtain repeatable results, even when conducted in a distant, second laboratory.

Another, more limited application of mtDNA sequence analysis to human evolution and history is also instructive, but ultimately baffling to the outsider because of still unresolved controversies. Anthropologists have been curious for some time about

the question of when the Americas were populated by humans and where did these people originate? Before the advent of molecular genetic analyses several time-honored approaches had suggested answers, which cannot be discussed here in detail. The more conventional archeological studies use the various artifacts (pottery, tools, weapons) to trace lineages based on designs, patterns, materials used, and so on. A more recent development is the application of the tools of linguistics to the comparative analysis of the languages spoken by the various native Americans (North and South America). Such an analysis had suggested three distinct linguistic divisions: Amerind (spoken by American Indians in North, Central, and South America), Eskimo-Aleut (spoken by the Inuit (Eskimo) and Aleutian Islanders), and Na-Dene (spoken by the by people of the Northeast Coast of Canada and the United States, as well as the Navajo and Apache, believed to have migrated south to their present location around 1000 BC). An interpretation was that there were three distinct waves of immigration across the Bering Straits, in agreement with other archeological data, such as distinctive features of molar shapes. mtDNA data assembled by Wallace, Torroni, and colleagues [reviewed in Reference 20] not only confirmed the Asian origin of the native Americans, but appeared to lend strong support to the three waves of migration hypothesized by the linguists. Four haplogroups were found, based on extensive restriction mapping, and the relative abundance of these groups could be interpreted in terms of three distinct migrations separated in time by thousands of years.

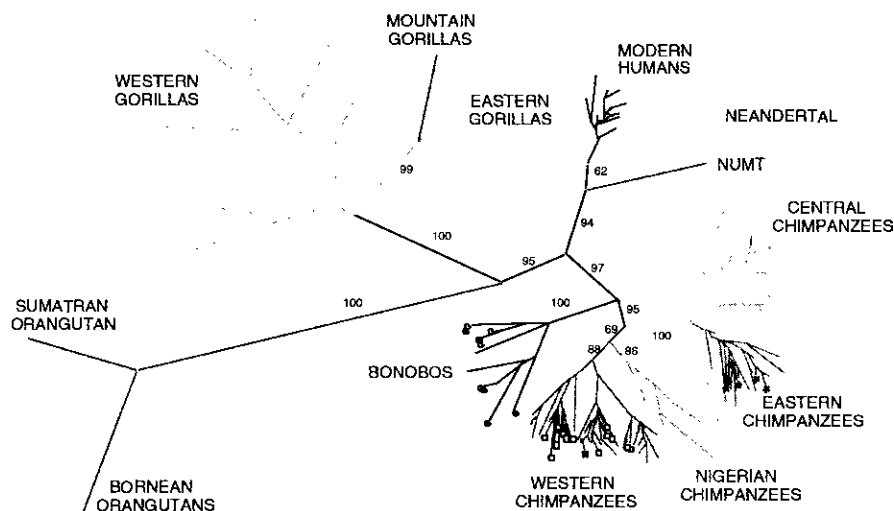
Somewhat ironically, a former student of Wallace has recently collected additional data that challenge the interpretation of his mentor [25]. The new findings of all four haplotypes among the three linguistic groups (mtDNA samples from 1300 American Indians) appear to fit better to a scenario in which a single wave of immigration brought women with all four haplotypes across the Bering Strait. Subsequent migrations, and the possibility of severe temporary reductions in one or more local populations due to environmental influences may have caused the loss of particular haplotypes (another bottleneck), and hence the current distributions.

### 8.3 PRIMATE EVOLUTION

Phylogenetic analysis of mtDNA sequences can of course be pushed back in time to include other species. No significant surprises about evolutionary history may be expected when comparing different vertebrates, or even different mammals. However, for calibrating molecular clocks, and for establishing more precisely the time of divergence of the nonhuman apes, a comparison of the mtDNA sequences of humans, chimpanzee, pigmy chimpanzee, gorilla, and orangutan has been very informative. Horai and colleagues [26] found these genomes to be highly homologous, with a synonymous substitution rate of  $3.89 \times 10^{-8}$  per site per year, if one assumes that the orangutan and the African apes diverged 13 million years ago. In the D-loop region this rate was almost twice as high. The analysis also included three human mtDNA sequences from Africans, Europeans, and Japanese to shed light on the origin of modern humans. From the estimated substitution rates the age of the last common ancestor of modern humans was calculated to be  $143,000 \pm 18,000$  years, and the hypothesis of the African origin of *Homo sapiens sapiens* received additional support.

The most detailed examination of the noncoding control region (human nucleotide positions 16,053–16,465) of the nine taxa of African or African-derived hominoids (humans, chimpanzees, bonobos and gorillas) has been undertaken by Gagneux and colleagues [27], who examined 1158 unique haplotypes, including 811 humans from around the world. A phylogenetic tree was constructed, which also included orangutans and the newly established sequence from *Homo neandertalensis* (Figure 8.1). For details on the computer analysis and related analytical methods the reader is referred to the original paper. The final tree shown was also subjected to various degrees of topiary pruning [11], but only one intermediate level is shown here. The tree illustrates in dramatic fashion some previous conclusions, and it adds some novel insights. It is clearly supportive of the closer relationship between humans and chimpanzees relative to the other great apes. Most remarkably, it reveals deep branches between chimpanzees in various African locations, corresponding to the existence of several subspecies and a previously unrecognized subclade of chimpanzees in Nigeria and northern Cameroon. There is significantly more variation between the major chimpanzee clades than between the entire human population. The appearance of the tree and specifically the relatively shallow human branches are consistent with the idea of a demographic bottleneck in human evolution; in other words, with the idea that the present human lineage arose from a small effective population of relatively recent origin. A mtDNA fragment that entered the nuclear genome was also included: it originated after the divergence of humans from the great apes, and even after the divergence of present humans from the Neandertals.

An interesting corollary to these studies was recently published by Kenyon and Moraes [28], who used human  $\rho^0$  cells (mtDNA-less) in fusions with enucleated cells from the hominoid apes—chimpanzee, pigmy chimpanzee, gorilla, and orangutan—to produce “xenomitochondrial hybrids.” Cybrids with chimpanzee or gorilla mtDNA



**Figure 8.1** Primate evolution based on the analysis of mtDNA. (From Reference 27, with permission.)

and a human nucleus could respire and carry out oxidative phosphorylation. By contrast, the orangutan mtDNA and the human nuclear genome were no longer compatible. The results support the notion that the nuclear and mitochondrial genomes have to coevolve at a significant number of loci in order to maintain the critical interactions between OXPHOS proteins encoded by the nucleus and proteins encoded by the mitochondria. Thus, the more closely related ape mt genomes could be functionally expressed to interact with human nuclear gene products, but the orangutan mt genome had evolved far enough, since the distant separation of the lineages, to yield mitochondrial proteins, which presumably could no longer interact with human proteins in the inner mitochondrial membrane. It will now be a challenge to distinguish simple polymorphisms with no structural-biochemical consequences from those nucleotide changes (and the resulting amino acid substitutions) that contribute to the defective interactions between nuclear and mitochondrial gene products. Unfortunately, the use of recombinant (human-primate) mtDNA molecules is technically still impossible. On the other hand, the available crystal structures for complexes III and IV may guide the search for protein-proteins interaction involving nuclear-coded and mitochondrial peptides, and perhaps reveal critical contact sites.

#### 8.4 HUMAN Y CHROMOSOME VARIATION

A crucial, inherent advantage of the mitochondrial genome in the analyses described above is the absence of recombination, facilitating the construction of phylogenies, and the estimation of the time intervals elapsed between the different branch points, assuming that the molecular clock is running at a constant rate. Another chromosome which has long been recognized not to participate in recombination is the Y chromosome. The statement has to be qualified, since a portion at the tip of the short arm, the pseudoautosomal region, does participate in chiasma formation and recombination. The male-specific portion of the Y chromosome, however, offers an alternative and complementary system for the study of human evolution and migratory pathways, and data on this chromosome are being collected and interpreted at an increasing rate. It is self-evident that the Y chromosome is inherited along the paternal lineage, since it is the male-specific sex chromosome containing the critical gene (SRY) encoding the testis determining factor [29]. By comparison with mtDNA the Y chromosome is huge, comprising approximately 60,000 kb (60 Mb) of DNA. The great bulk of Y chromosome DNA, especially the long arm (Yq), is made up of constitutive heterochromatin consisting of various families of repetitive DNA with no obvious genetic information. It has taken some time to define unique loci and to establish polymorphisms at these loci in human populations [30–35].

A detailed discussion of human Y chromosome polymorphisms would go beyond the scope of this monograph. On the other hand, it is worthwhile to consider how far the deductions from the analysis of mtDNA have been confirmed by similar studies with Y chromosome sequences, and what kinds of unresolved discrepancies exist. Independent confirmations of the reconstruction of human evolution are of course a source of intellectual satisfaction and they strengthen the conclusions drawn. Discrep-

ancies, on the other hand, raise issues about methodology, interpretation, limitations of computer analyses, but in the long run they can lead to new insights and progress.

A comprehensive and recent description of the geographic distribution of human Y chromosome variation can be found in the publication by Hammer and colleagues [35], which includes data from 1500 males from 60 population groups, subdivided into 15 major groups based on geographic, linguistic, and historical information. In agreement with other genetic analyses, and specifically the mtDNA data, the Y-chromosome haplotype distributions show that sub-Saharan African populations are distinct from the rest of the world, and they exhibit the highest within-group diversity. Globally, the variants found outside of Africa represent subsets of the haplotypes found within Africa. Hammer and colleagues are careful to point out that while such a within-group and among-group pattern also supports the hypothesis of the origin of modern humans from an older African population, with migrations of subgroups leading to the population of distal sites, and more limited genetic diversity elsewhere resulting from bottlenecks, it may nevertheless be an overly simplistic view. In the absence of additional information, the genetic data are equally consistent with multiple short-range and long-range migrations, genetic drift due to founder effects and small population sizes (near extinction). Africa may well have been the origin of more than one migration originating from different parts of Africa. Notably, the Y chromosome data also are consistent with the relatively recent (200,000 years) origin of our common ancestors, but may be a consequence of a small effective population size throughout the Late Middle Pleistocene, rather than a true definition of the period of origin of *Homo sapiens*. Additional theoretical and methodological considerations can be found in a paper by Hammer and coworkers [35], who also cite numerous recent publications.

## 8.5 FORENSIC APPLICATIONS

Under other monarchies the male line takes precedence of the female in tracing genealogies, but here the opposite is the case—the female line takes the precedence. Their reason for this is exceedingly simple, and I recommend it to the aristocracy of Europe: They say it is easy to know who a man's mother was, but, etc., etc.

Mark Twain: "Letters from the Sandwich Islands"  
(describing the Hawaiian Monarchy)

Mitochondrial DNA sequences also mutate rapidly enough to create distinctions (polymorphisms) between members of the same ethnic group, that is, close "relatives" on an evolutionary scale, though not relatives in the legal sense. Applications in forensic investigations have been made, and two such applications are used here as examples to illustrate the methodology and the potential limitations.

Attention is focused on the mtDNA control region (D loop). It was first found in 1983, in a relatively small sample of Caucasians and Africans, that there was ~1.7% nucleotide diversity in ~900 base pairs, with most of the differences clustered in two hypervariable regions [36]. The 347 base-pair hypervariable region (within nu-



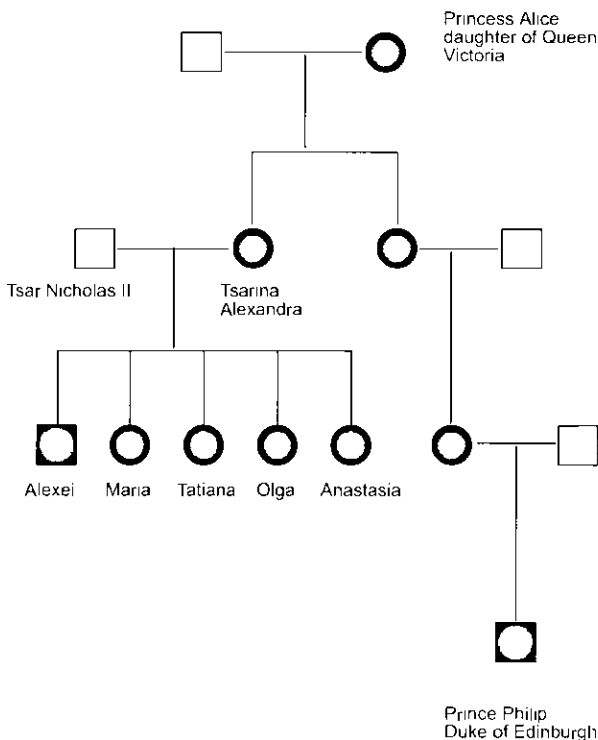
cleotides 16,023–16,388) was later analyzed by Orrego and King in 10 additional unrelated Caucasian individuals of European ancestry. In the combined dataset from Greenberg and colleagues [36], Anderson and colleagues [37] and Orrego and King, 32 of 347 sites compared were found to be variable, and pairwise comparisons showed differences ranging from 1 to 13 nucleotides (average: 5.9). A formal statistical analysis revealed that the observed and expected number of sequence differences between pairs of individuals closely approximated a Poisson distribution, which allows one to calculate that the probability of two unrelated Caucasian individuals having an identical sequence of 347 base pairs is 1 in 370 [see Reference 38 for a discussion of these results and related issues].

With oligonucleotide primers corresponding to the conserved flanking sequences (tRNA<sup>Pro</sup> and tRNA<sup>Phe</sup>) it is a routine matter to amplify the D-loop region from very small tissue samples. As described by King [38], a second amplification with nested but unequal amounts of primers can yield a single-strand product ready for DNA sequencing. Optimization of conditions to reduce artifacts from polymerase errors is part of the standard methodology, and mosaics created with unintended target sequences can be detected and eliminated because the normal target is so well known. A successful test analysis was performed with mtDNA from a baby tooth extracted 20 years earlier, and the sequence matched to mtDNA from the now adult, his sister, and his mother. Baby teeth may be one of the best ways to preserve DNA samples for posterity and future cloning, should that become socially acceptable.

The technology was subsequently applied to a problem illustrating in a marvelous and touching way the application of science to seek justice and uphold human rights. An Interamerican Commission on Human Rights, upon a request by the National Commission on the Disappearance of Persons formed by the newly elected democratic government of Argentina in 1983, assisted the Grandmothers of the Plaza de Mayo and other surviving relatives to be reunited with their kidnapped children. These children had been born to couples and women who subsequently “disappeared,”—murder victims of the military junta that ruled Argentina from 1976 to 1983. The children were often given away or adopted by the military or police, but Argentinian courts were willing to promote a reunification of the families, if objective scientific proof could establish the identity of these children. Since mtDNA is maternally inherited, haplotyping of mtDNAs from the children and relevant surviving relatives and specifically maternal grandmothers became a legally accepted method to test kinship in the absence of surviving parents.

While the science was objective and frequently decisive in relating children to grandmothers, a host of legal, ethical, sociological, and psychological problems arose because, by the time the tests became possible, the children were no longer small, they had developed relationships with their foster parents, and it was not always very clear who had the right to know, and what to do with the new knowledge. Should a child be informed that the foster parents were among the killers of their parents? Should they be coerced back to their original families? Even the political climate has changed in Argentina and the judicial system is no longer so supportive of the Grandmothers. As King points out, the average age of the “children” is now [1997] 21 years, and they will obtain the legal right to find out their identities if they wish.

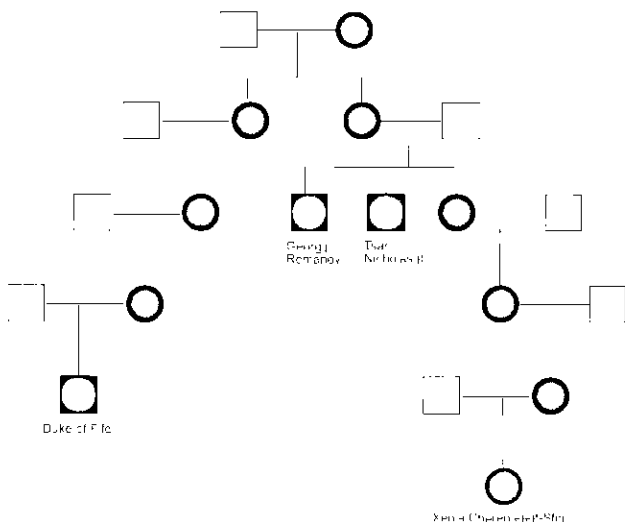
Another headline-producing story involving mtDNA sequencing was the recent authentication of the remains of the Tsar Nicholas II of Russia, his wife and children, and some of his closest servants [39,40]. The Russian imperial family had been executed during the revolution and buried in a mass grave in the vicinity of Yekaterinburg. Nine sets of skeletal remains were recovered in 1991, but it remained to prove that these indeed included the remains of the Tsar, his wife, and three of their children. Tissue from the bones yielded sufficient mtDNA to allow amplification and sequencing of D loops, to be compared with those of living and dead relatives (Figure 8.2). The Tsar's brother was exhumed from his grave in St. Peter and Paul Cathedral



**Figure 8.2** Pedigrees of European aristocracy showing maternal inheritance of mtDNA polymorphisms. (Adapted from Reference 39)

in St. Petersburg where he had rested since 1899. Prince Philip, a known maternal relative, had mtDNA that matched that of the presumed Tsarina. Sequences of the Tsar matched those of two living maternal relatives, but in an additional twist to the story it was found that the Tsar was heteroplasmic at position 16,169 (72%C/28%T). There were suspicions of contamination after the first publication which could be removed by independent repeats of the sequencing, and by the finding that the Tsar's brother was also heteroplasmic at the same position (38%C/62%T). Countess Xenia Cheremeteff-Sfiri and the Duke of Fife were homoplasmic, but had the same sequence at the other six positions where substitutions relative to the standard reference sequence had been found (Figure 8.3). The existence of heteroplasmy in two siblings (but with different ratio), and the progression to homoplasmy within four generational events is another interesting example illustrating rapid mtDNA segregation. It adds evidence in support of the bottleneck mechanism (see Chapter 7).

The use of mtDNA polymorphisms in forensics has attracted the attention of the FBI and the US Military (the Armed Forces DNA Identification Laboratory in Rockville Maryland). Thus, a considerable number mtDNA control region sequences from hundreds of individuals representing more than 120 families have been analyzed recently, and the results have caused surprise and consternation. Contrary to expectations and previous estimates, the mutation rate was much larger than expected. Instead



**Figure 8.3** Pedigrees of European aristocracy showing maternal inheritance of mtDNA polymorphisms. (Adapted from Reference 40)

of a rate of 1 mutation every 12,000 years (~600 generations), deduced from the differences between the great apes and humans and their divergence ~5 million years ago, the observed differences among contemporary humans yielded an estimate of 1 mutation every 800 years. The controversy received fuel from both additional supporting studies, and from studies failing to confirm the high mutation rate [see Reference 41 for further discussion]. It is clear that the acceptance of a much higher mutation rate would also upset various estimates of landmarks in human evolution (emergence from Africa, expansion of modern Europeans, immigration into North America).

More data will resolve whether the discrepancy is due to "statistical fluctuations" (low sample size). There is also debate about the existence and meaning of "hot spots." If hot spots exist, mutations will be observed over the short term, but over the long term such mutations can revert back to the original sequence, in effect giving an average long-term mutation rate that is significantly slower. Another issue to be resolved is whether the DNA in the control region is not subject to selection pressure. Since it is noncoding, it has been assumed that mutations in this region are neutral. On the other hand, such mutations may in subtle ways affect the initiation of transcription and replication of mtDNA, even if none of the identified sequence elements for protein binding are directly involved.

## 8.6 FUTURE CHALLENGES

The molecular analysis of mtDNA has progressed from restriction mapping to sequence analyses, and the available database has grown substantially over the past decade. In the future, sequence comparisons will be significantly expanded from the D-loop region to individual mitochondrial genes. All of these studies include attempts to test and refine the molecular clock, and to determine whether the clock runs at different speeds for different segments of the mitochondrial genome [see Reference 26 for an illustration]. Regions with a high degree of sequence conservation will attract attention; this may include not only structural genes, but also conserved regions in the D loop, which have not yet been identified as conserved binding sites of transcription factors, and other domains important for DNA replication and maintenance.

It is satisfying that the conclusions derived from mtDNA are already largely compatible with an accumulation of new data for loci on the Y chromosome, providing the mitochondrial Eve with a Y chromosome-bearing Adam from the same geographical area. But will such sequence comparisons ever be definitive in excluding any interbreeding between the Neandertals and our *Homo sapiens sapiens* ancestors, for example? Will there ever be sufficient data for phylogenies that uniquely identify the relationship between ethnically diverse human groups, and are capable of settling questions about the human migrations across the Bering Strait and the peopling of the Americas? Even if mtDNA analysis cannot answer all of these and other questions, it is clear that this molecule has provided the stimulus and the raw data for a very impressive and successful intellectual effort in understanding human evolution.

The data are also available to push evolutionary studies of mtDNA back in time to include the higher primates. The constraint imposed by nuclear genes on the evolu-

tion of mitochondrial genes has become apparent from a functional test of mtDNA of the great apes in the presence of a human nuclear genome (xenomitochondrial hybrids). Where, precisely, is the incompatibility of human nuclear genes with orangutan mtDNA? Is only a small number of nucleotide changes, or even a single nucleotide change, critical?

Forensic applications have been illustrated with some spectacular and widely publicized examples. The challenge in the future will be to find "cases" of sufficiently broad interest to warrant publication of the results in journals like *Nature*, since the technology appears to be on a firm footing. Heteroplasmy and rapid fluctuations in the proportion of genomes may add some complications in specific situations.

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# 9

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## MITOCHONDRIA AND PHARMACOLOGY

### 9.1 INTRODUCTION

Studies on mitochondria have for a long time benefited from the judicious use of specific drugs, antibiotics, inhibitors and/or analogues, which have helped in defining pathways, or in focusing on a particular complex, or even in creating phenocopies of specific mutations. Most of these have been introduced in previous chapters in the context of the reactions or complexes being discussed. The following discussion is intended to be a summary and compilation for easy reference and identification of the targets of both the common and less common drugs being employed in this field. Drugs that inhibit specific enzymes in biochemical pathways and metabolic cycles (e.g., fluorocitrate) are not covered exhaustively, unless there is a special relevance to mitochondrial function.

One can subdivide the collection of drugs/inhibitors into several categories. A summary of information with some relevant, recent references is presented in Table 9.1. No attempt is made to be exhaustive or to identify the initial discovery of the inhibitor and its specificity.

TABLE 9.1

| Category (Name)                                                           | Target      | Reference | Discussion in this Book |
|---------------------------------------------------------------------------|-------------|-----------|-------------------------|
| 1. Inhibitors of Electron Transport                                       |             |           |                         |
| Rotenone                                                                  | Complex I   | 1–6       | 5.2.2.1<br>Figure 5.10  |
| Amytal                                                                    |             |           |                         |
| Piericidin A                                                              |             |           |                         |
| <i>N</i> -Methylquinolinium derivatives                                   |             |           |                         |
| <i>N</i> -Methylpyridinium derivatives (MPP <sup>+</sup> )                |             |           |                         |
| Others                                                                    |             |           |                         |
| Malonate                                                                  | Complex II  |           |                         |
| Antimycin A                                                               | Complex III | 7, 8, 8   | 5.2.2.3<br>Figure 5.15  |
| Quinoline <i>N</i> -oxides                                                |             |           |                         |
| Myxothiazol                                                               |             |           |                         |
| Azide, carbon monoxide, cyanide                                           | Complex IV  |           |                         |
| 2. Uncouplers/Ionophores                                                  |             |           |                         |
| Dinitrophenol                                                             |             |           | 5.4                     |
| Valinomycin                                                               |             |           |                         |
| Carbonylcyanide- <i>p</i> -trifluoromethoxy phenylhydrazone (FCCP)        |             |           |                         |
| 3. Inhibitors of ATP Synthase                                             |             |           |                         |
| Oligomycin B                                                              | Complex V   | 9–11      | 5.5.4                   |
| Dicyclohexylcarbodiimide (DCCD)                                           | Complex V   |           |                         |
| Venturicidin                                                              | Complex V   |           |                         |
| 4. Inhibitors of Transport Systems                                        |             |           |                         |
| Atractyloside                                                             | ANT         | 12, 13    | 5.6.4.1                 |
| Bongkreikic acid                                                          |             |           |                         |
| 5. Sensors of Membrane Potential                                          |             |           |                         |
| Rhodamine123                                                              |             | 14, 15    | 5.4.3<br>Figure 5.22    |
| 3,3′ Dihexyloxacarbocyanine iodide                                        |             |           |                         |
| DiOC <sub>6</sub> (3)                                                     |             |           |                         |
| 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine (JC-1) |             |           |                         |
| Chloromethyl-X-rosamine (CMXRos)                                          |             |           |                         |

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## GLOSSARY

- active transport** transport of ions across a membrane against a gradient driven by ATP hydrolysis
- adenine nucleotide translocase (ANT)** ADP/ATP translocator (exchange protein) in inner mitochondrial membrane
- AD** Alzheimer's disease
- ADP** adenosine diphosphate
- AIF** apoptosis-inducing factor
- amphipathic** molecule with distinct hydrophobic and hydrophilic domains
- ANT** adenine nucleotide transporter or translocase
- antigenic determinant** a short segment of a peptide (terminal or internal) that is recognized by a monoclonal antibody; an antigenic determinant may also be formed in the tertiary structure by the spatial juxtaposition of noncontiguous side chains
- antiport** Permease-mediated diffusion of two solutes in opposite direction; participation of both is obligatory
- apoptosis** programmed cell death
- ATP** adenosine triphosphate
- bilayer** phospholipid bilayer of biological membranes
- caspase** A family of proteases activated in a cascade during apoptosis
- CAP** abbreviation for chloramphenicol; or, catabolite gene activating protein
- cell fusion** fusion of plasma membranes of two or more (somatic) cells creating a (binucleate cell) hetero- or homokaryon that may develop into a hybrid cell

- cell hybrid** somatic cell created from the fusion of two cells from the same or different species after all the chromosomes have been combined in a single nucleus
- chaperone** a protein that can associate with other unfolded or misfolded proteins and assist in the proper folding and assembly into quaternary complexes
- chemiosmotic hypothesis** a proton gradient (an electrochemical gradient) established by electron transport is driving oxidative phosphorylation; the electron transport complexes and the ATP synthase are in physically distinct regions of the membrane
- CIFA** cytochrome c-inactivating factor of apoptosis
- CMS** cytoplasmic male sterility
- cofactor** small organic molecule complexed with a protein and participating in enzyme catalysis
- complex I** NADH-CoQ oxidoreductase or NADH-ubiquinone oxidoreductase
- complex II** succinate-CoQ oxidoreductase or succinate-ubiquinone oxidoreductase
- complex III** CoQ-cytochrome c oxidoreductase
- complex IV** cytochrome c-oxidase
- consensus sequence** a short sequence of nucleotides defining a binding site for a transcription factor; small deviations from the consensus sequence may be found in different genes and in different organisms
- COX** cytochrome oxidase (complex IV)
- cristae** foldings of the inner mitochondrial membrane (seen by electron microscopy) to enlarge the surface area
- cybrid** a hybrid cell created by the fusion of a nucleated cell and an enucleated cell; the enucleated cell typically contributes the mitochondria; the nucleated cell may or may not have mtDNA
- cytochrome** protein with heme as a prosthetic group
- cytoplasmic male sterility** plant phenotype: a defect in pollen production due to mutations in mtDNA or mitochondrial plasmids
- cytoskeleton** intracellular filamentous or tubular structures formed by actin, tubulin or intermediate filament proteins used to organize the cytoplasm
- D loop** a region of vertebrate mtDNA that is noncoding, but contains promoters, an origin of mtDNA replication; a temporary arrest in DNA elongation creates a "displacement loop" shortly after initiation of replication;
- DMA** dimethylallyl pyrophosphate
- dominant mutation** a mutation that leads to a mutant phenotype in the presence of a normal (wild-type) allele
- dynein** an ATP-dependent molecular motor moving along microtubules towards the minus end
- EEG** electroencephalograph (abnormalities)
- electron transport chain** a series of complexes in the inner mitochondrial membrane to conduct electrons from the oxidation of NADH and succinate to oxygen

**elongation factor** factor required for elongation of a polypeptide by the addition of amino acids and formation of new peptide bonds

**epitope** antigenic determinant

**EPR** electron paramagnetic resonance (formerly electron spin resonance)

**ER** endoplasmic reticulum

**ETC** electron transport chain

**ETF** electron transfer flavoprotein

**facilitated diffusion** diffusion of small molecule across a membrane with the help of a carrier protein

**FPP** farnesyl pyrophosphate

**gametogenesis** formation of spermatozoa or ova (oocytes) involved in sexual reproduction

**germline** a group of cells set aside early in development giving rise to the ovary or testis and the tissues responsible for gametogenesis

**GFP** green fluorescent protein

**GPP** geranyl pyrophosphate

**GSH** glutathione

**HD** Huntington's disease

**heteroplasmy** a population of mitochondrial DNAs in the same cell with two or more distinguishable polymorphisms or mutations present in significant proportions

**high-energy bond** a chemical bond that may be hydrolyzed with the release of a substantial amount of free energy; a group involved in such a bond may be readily transferred from the donor to another acceptor. Example: phosphate transfer from ATP to small molecules or to amino acid side chains in proteins

**high-energy phosphate** a relative term, reflecting the free energy change associated with the hydrolysis of the compound X-O-P to form X-OH and inorganic phosphate; an acyl phosphate is higher than a phosphomonoester

**HMG** hydroxymethylglutaryl

**homoplasmy** a population of mitochondrial DNAs with no significant polymorphisms

**HSP** heavy-strand promoter

**hybrid cell** see cell hybrid

**hydrogenosome** an organelle believed to be related to mitochondria that has lost all of its genome, and can no longer respire; it represents an extreme evolutionary development of mitochondria in organisms that have become adapted to an anaerobic environment

**hydrophilic** soluble in water; readily hydrated; a polar molecule capable of forming hydrogen bonds

**hydrophobic** insoluble in water, incompatible with aqueous surroundings; lipophilic

**ICE** interleukin-1 $\beta$ -converting enzyme

- IMP** integral membrane protein
- initiation factor** proteins required for initiation of protein synthesis at the 5' end of a messenger RNA (mRNA)
- integral membrane protein** protein with at least one transmembrane segment; detergents are required for solubilization
- intermembrane space** space between inner and outer membranes
- ionophore** small peptide or organic molecule capable of short circuiting (permeabilizing) a membrane for specific ions (facilitated diffusion)
- ion pump** integral membrane protein that can pump ions against a gradient coupled to the hydrolysis of ATP
- IPP** isopentenyl pyrophosphate
- IRE** iron-responsive element
- iron-sulfur center** nonheme, inorganic iron ion complexed with cysteine chains, inorganic sulfide, making a protein capable of conducting electrons in electron transport or redox reactions
- IRP** iron-responsive protein
- kinetoplast** specialized mitochondrion in protozoa such as the trypanosomatids located adjacent to the basal body of the flagellum
- kinesin** an ATP dependent molecular motor that can move vesicles/particles along microtubules towards the plus end
- Krebs cycle** tricarboxylic acid cycle in the mitochondrial matrix
- KSS** Kearns-Sayre Syndrome
- LDL** low-density lipoprotein
- LHON** Leber's hereditary optical neuropathy
- LSP** light-strand promoter
- mdm* mutants** yeast mutants defective in mitochondrial distribution and morphology
- mas* mutants** yeast mutants defective in mitochondrial protein import
- matrix** space enclosed by the inner mitochondrial membrane
- MD ring** mitochondrion-dividing apparatus
- megapore** leakage channel thought to be responsible for the mitochondrial permeability transition
- MELAS** mitochondrial encephalomyopathy with lactic acidosis and strokelike episodes
- MEM** mitochondrial encephalomyopathies
- membrane potential** combination of proton and ion gradients across the inner membrane making the inside negative relative to the outside
- MERRF** a mitochondrial disease including myotonus, epilepsy, ragged red fibers; a combination of abnormalities resulting from a mitochondrial mutation
- MF** microfilament

**mF** mitochondrial fusion

**MIMyCa** maternally inherited disorder with adult-onset myopathy and cardiomyopathy

**mitochondrial plasmid** linear or circular small DNA present in addition to the mitochondrial genome

**mitoplast** mitochondrion without outer membrane

**mobile carrier** small molecule such as coenzyme Q (ubiquinone) shuttling electrons between complexes in the mitochondrial electron transport chain

**MPT** mitochondrial permeability transition

**MT** microtubule

**mtDNA polymorphism** existence of mtDNAs in a species differing in size (deletions, insertions) or in single nucleotides (missense mutations)

**mtDNA** mitochondrial DNA

**NADH** nicotinamide-adenine dinucleotide (reduced form)

**NARP** neuropathy, ataxia, retinitis pigmentosa

**ND1** A subunit of complex I of the mitochondrial electron transport chain; one of seven ND subunits encoded by the mammalian mtDNA

**nDNA** nuclear DNA

**Nebenkern** an aggregation of mitochondria next to the nucleus found at a specific developmental stage in the formation of spermatozoa in *Drosophila* and other insects

**nonmendelian inheritance** cytoplasmic inheritance; genes in mitochondria or chloroplasts

**NRF** nuclear respiratory factor

**NRK** normal rat kidney

**ORF** open reading frame in a cDNA or mRNA

**OXPHOS** oxidative phosphorylation

**PCR** polymerase chain reaction

**PD** Parkinson's disease

**peripheral membrane protein** protein associated with membrane via protein-protein interactions; solubilized by extremes of salt or pH

**permeability transition** collapse of membrane potential due to large non-specific leakage channel in inner membrane

***pet* mutants** yeast mutants with defects in respiration or oxidative phosphorylation forming small (petite) colonies under certain conditions

**PG** phosphatidylglycerol

**PI** phosphatidylinositol

**plasmid** a linear or circular DNA capable of an autonomous existence in an organism or an organelle; can replicate and maintain itself without integration into the genome

**polytopic membrane proteins** integral membrane proteins with multiple trans-membrane segments

**porin** a pore-forming protein in the outer mitochondrial membrane

**posttranslational modification** modification of proteins by phosphorylation, proteolytic cleavage, or other covalent changes involving side chains or the termini

**programmed cell death** cell death brought about by a stimulus triggering a series of regulated events leading to nuclear fragmentation, chromatin fragmentation, protein turnover, the mitochondrial permeability transition, and death of the cell

**PT** permeability transition

**reactive oxygen species (ROS)** peroxide, peroxide radical, and hydroxyl radical with high reactivity toward DNA, protein, and lipids

**restriction fragment length polymorphism** variation in the length of restriction fragments detected by a specific probe due to presence or absence of a restriction site (RFLP)

**rho<sup>0</sup> mutants** cells with mitochondria but no mtDNA

**R loop** a smaller loop at the origin of mtDNA replication formed by displacement of a DNA strand by an RNA serving as primer

**RNA editing** changes in RNA sequence by insertion, deletion or base modification after synthesis of the original transcript encoded by DNA sequence

**ROS** see reactive oxygen species

**RFLP** see restriction fragment length polymorphism

**SDH** succinate dehydrogenase, an enzyme of the TCA cycle associated with complex II

**senescence** inability to continue to undergo mitotic cell divisions in an aging population of cells

**shuttle** a combination of reactions outside and inside the mitochondria for transporting a metabolite in and out of the organelle; the overall shuttle mechanism also requires a specific carrier in the membrane

**signal sequence** N-terminal sequence for targeting proteins into mitochondria

**SNP** single nucleotide polymorphism

**SOD** superoxide dismutase

**state 3 respiration** respiration in tightly coupled mitochondria, when oxygen consumption depends on availability of ADP and P<sub>i</sub>

**state 4 respiration** mitochondria in absence of ADP

**sublimons** rare mtDNA molecules found at substoichiometric levels; may be products of aberrant recombination

**substrate level phosphorylation** transfer of a high energy phosphate from an intermediate to ATP

**Southern blot** detection of separated restriction fragments on filter paper, usually by a radioactive probe

**symport** permease-mediated diffusion of two solutes in the same direction; both solutes must be present

**TCA cycle** tricarboxylic acid cycle, also known as Krebs cycle

**TFA** transcription factor A in mammalian mitochondria

**thermogenesis** production of heat from metabolism in warm-blooded animals

**Tim** protein complex in inner mitochondrial membrane required for protein import

**TIRS** terminal inverted repeat sequences

**Tom** protein complex in outer mitochondrial membrane required for protein import

**topogenesis (protein)** pathway or mechanism for directing a protein to its final destination and orientation in a specific cellular membrane

**transcription factor** protein required for initiation of transcription by RNA polymerase at specific sites; a regulatory factor in gene expression (TFA)

**transposon** also: transposable element; a short segment of DNA that can be mobilized to move around in the genome; it encodes some of the enzymes needed for such relocations

**uncoupler** protein or other molecule capable of uncoupling electron transport from oxidative phosphorylation

**Western blot** immunochemical detection of proteins on a nylon or paper filter after separation (usually by electrophoresis)

**wild-type** normal form of an organism in a natural (wild) environment

**UCP-1** uncoupling protein; first discovered in brown adipose tissue; other, closely related members of this class of proteins (UCP-2, etc.) have been discovered

**URF** unidentified reading frame

**UTR** untranslated region of mRNA at 5' or 3' end

**VER** visual evoked response (abnormalities)



# INDEX

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